Acid β-Glucosidase: Enzymology And Molecular Biology of Gaucher Disease

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ABSTRACT

Human lysosomal β-glucosidase (D-glucosyl-acylsphingosine glucohydrolase, EC 3.2.1.45) is a membrane-associated enzyme that cleaves the β-glucosidic linkage of glucosylceramide (glucocerebroside), its natural substrate, as well as synthetic β-glucosides. Experiments with cultured cells suggest that *in vivo* this glycoprotein requires interaction with negatively charged lipids and a small acidic protein, SAP-2, for optimal glucosylceramide hydrolytic rates. *In vitro*, detergents (Triton® X-100 or bile acids) or negatively charged gangliosides or phospholipids and one of several "activator proteins" increase hydrolytic rate of lipid and water-soluble substrates. Using such *in vitro* assay systems and active site-directed covalent inhibitors, kinetic and structural properties of the active site have been elucidated. The defective activity of this enzyme leads to the variants of Gaucher disease, the most prevalent lysosomal storage disease. The nonneuronopathic (type 1) and neuronopathic (types 2 and 3) variants of this inherited (autosomal recessive) disease but panethnic, but type 1 is most prevalent in the Ashkenazi Jewish population. Several missense mutations, identified in the structural gene for lysosomal β-glucosidase from Gaucher disease patients, are presumably casual to the specifically altered post-translational oligosaccharide processing or stability of the enzyme as well as the altered *in vitro* kinetic properties of the residual enzyme from patient tissues.

INTRODUCTION

The first description of Gaucher disease was by Philip Gaucher in his medical thesis, "De l'epithelioma primitif de la rate" in 1882. Based on the finding of unusual large foamy cells, he thought this was a primary neoplasm of the spleen. Subsequent research characterized lipid abnormalities, related enzymology, the autosomal recessive inheritance, and molecular biology of Gaucher disease. Gaucher disease was the first lysosomal storage disease described, was the fist such disease to have its etiologic enzymatic defect defined,[1,2] and is the most prevalent lysosomal storage disease. Worldwide an estimated 20,000 to 30,000 individuals are affected with Gaucher disease. The disease is characterized by remarkable heterogeneity of the clinical phenotypes within and among the three major delineated types (Table 1). The disease is now defined by the accumulation of glucosylceramide in tissues (Figure 1) in conjunction with either the defective activity of the lysosomal hydrolase, acid β-glycosidase (EC 3.2.1.45), or, as has occurred in a single patient, the deficiency of SAP-2, a naturally occurring protein activator of acid β-glucosidase. This review emphasizes the normal enzymology and molecular biology of acid β-glucosidase and SAP-2 as a basis for understanding the corresponding abnormalities in the Gaucher disease variants.

I. SYNTHESIS AND SOURCES OF GLUCOSYLCERAMIDE

The major natural substrate for acid β-glucosidase is *N*-acylsphingosyl-1-*O*-β-D-glucoside (Figure 1). This compound also has been called: glucosylceramide, ceramide β-glucoside, or glucocerebroside and the enzyme has been referred to as glucosylceramidase, ceramide β-glucosidase, glucocerebrosidase, or acid β-glucosidase. With some variation (see below) in chain length, the natural sphingosyl moiety is (2**S**,3**R**,4**E**)-2-amino-4-octadecene-1,3-diol.[3,4] Glucosylceramide is structurally similar to galactosylceramide (also called galactocerebroside or cerebroside), which is present as an anabolic end product in the nervous system, mostly in the myelin sheath, but is practically absent from most other tissues. In comparison, glucosylceramide is widely distributed in many mammalian tissues in rather small quantities as a metabolic intermediate in the synthesis or degradation of the more complex glycosphingolipids, such as the gangliosides or globoside.

Glucosylceramide is synthesized from ceramide and UDP-glucose.[5,6] A partially purified enzyme was used to condense ceramide and UDP-[^{14}C]glucose (Figure 1); the reaction had an optimal pH at 7.8 and K_m values of 0.12 and 0.8 m*M* for ceramide and glucose, respectively. Radin et al.[7] showed that in brain this enzymatic activity is localized to neurons and subsequent studies[8,12] demonstrated this pathway in brain microsomal preparations. Both hydroxy and nonhydroxy fatty acylsphingosines served as substrates.[13] The latter finding indicates the poor specificity of the *in vitro* assay, since most glucosylceramide and its higher derivatives contain nonhy-

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droxy fatty acids. Curtino and Caputto[14,15] proposed an alternative synthesis, i.e., that of glucosylsphingosine ("glucopsychosine") condensing with acyl coenzyme A (Figure 1). A third pathway proposed by Raghavan et al.[16] is the reversal of the acid β-glucosidase reaction. Thus, the "true" pathway of glucosylceramide biosynthesis under physiological conditions still awaits elucidation.

Glucosylceramide is also an intermediate in the degradative pathway of most complex glycosphingolipids. Except for galactosylceramide, sulfogalactosylceramide, and galactosyl-α-galactosyl-β-ceramide (found in tissues of patients with Fabry disease[17]) all glycosphingolipids have a lactosyl ceramide structure, i.e., ceramide-β-galactose (or galactosyl-β-glucosyl-β-ceramide) to which are linked α-galactose, *N*-acetyl neuraminic acid, *N*-acetylgalactosamine, and more complex carbohydrate combinations. The catabolism of these respective glycosphingolipids, which occurs in the lysosomes by a series of glycohydrolases having acidic pH optima, is exemplified in Figure 2, which shows pathways of degradation of GM_1-ganglioside and globoside. With either compound, glucosylceramide is an intermediate that upon further degradation by the acid β-glucosidase produces ceramide, the latter being further degrated by acid ceramidase[18,19] to sphingosine and fatty acid. The glucosylceramides from visceral sources (spleen, liver, kidney, and red blood cells) contain C22 to C24 fatty acid moieties predominantly, whereas those from brain of normals or Gaucher disease type 2 patients contain C18 (stearic) acids.[20,21] Similarly, the glucosylceramides from visceral tissues have a greater proportion of d18:1 spingosine bases, whereas those derived from brain gangliosides are enriched in eicosphingenine (d20:1).[22] The major source of glucosylceramide in the visceral tissues derives from degradation of leukocyte membranes.[23] There are numerous papers describing the properties, isolation, purification, and role of the respective glycohydrolases in normal animals or humans as well as the lipid storage disorders (summarized in the book by Kanfer and Hakomori[24]).

As described in detail in this review, the acid β-glucosidase has been studied extensively in extracts of numerous tissues and has been purified to homogeneity. *In vitro* the enzyme splits the β-glucosidic linkage forming glucose and ceramide. *In vivo* this reaction, of course, takes place continuously in the lysosomal compartment of intact cells, and attempts have been made to administer labeled glucosylceramide into cultured cells — especially skin fibroblasts; these have resulted in very low or no degradation of the externally added substrate by the intact cells.[25,26] In most of these experiments, the glucosylceramide was added to the growth media of the cells containing fetal calf serum. Most probably a complex was formed between the glucosylceramide and the albumin of the serum, from which molecules of the former were released, associated with the plasma membrane of the cells, and thereby introduced into the cell interior. In a different approach (Gatt et al., in preparation), glucosylceramide (labeled with the fluorescent probe, pyrene) was co-sonicated with spingomyelin or phosphatidylcholine and the small, unilamellar vesicles thus formed were coated with apolipoprotein E. When added to skin fibroblasts, the liposomes were taken up by receptor-mediated endocytosis utilizing the receptor for this apoliproprotein for internalization and transport to the lysosomes where they were efficiently degraded. in similar experiments, several glycosphingolipids (e.g., lactosylceramide, trihexosylceramide, G_{M3} and G_{M1} gangliosides, all labeled with pyrene as a fluorescent marker) was similarly administered into normal skin fibroblasts where they were efficiently degraded to ceramide. In the latter experiments there was no accumulation of glucosylceramide as intermediate, suggesting that acid β-glucosidase is not limiting in the sequential degradation, to ceramide, of glycosphingolipids administered into the intact lysosomes of cultured skin fibroblasts.

Table 1
Gaucher Disease — Clinical Types

Clinical features	Type 1	Type 2	Type 3
Onset	Child/adult	Infancy	Juvenile
Hepatosplenomegaly	+	+	+
Hypersplenism	+	+	+
Bone crises/fractures	+	−	+
Neurodegenerative Course	−	+ + +	+ +
Death	Childhood/ adulthood	By 2 years	2nd/4th decade
Ethnic predilection	Ashkenazi Jewish	Panethnic	Swedish

II. CHARACTERIZATION OF THE GLUCOSYLCERAMIDE CLEAVAGE COMPLEX

Since the demonstration of an *in vitro* glucosylceramide cleaving enzyme in mammalian tissues,[1,2] it has become increasingly clear that additional protein components, activator(s), may be necessary for the *in vivo* degradation of this glycolipid to ceramide.[27-31] In Section II.A, the purification methods for and properties of acid β-glucosidase are reviewed. In Section II.B a similar review is presented for two protein "activators". The interactions and functional relationships of acid β-glucosidase and these activators are detailed in Section IV.C.

A. Acid β-Glucosidase

1. Purification of Acid β-Glucosidase

Several methods have been developed for the purification of acid β-glucosidase from a variety of species, although human tissues have been the primary source for many investigations.[32-38] Early attempts to purify acid β-glucosidase from

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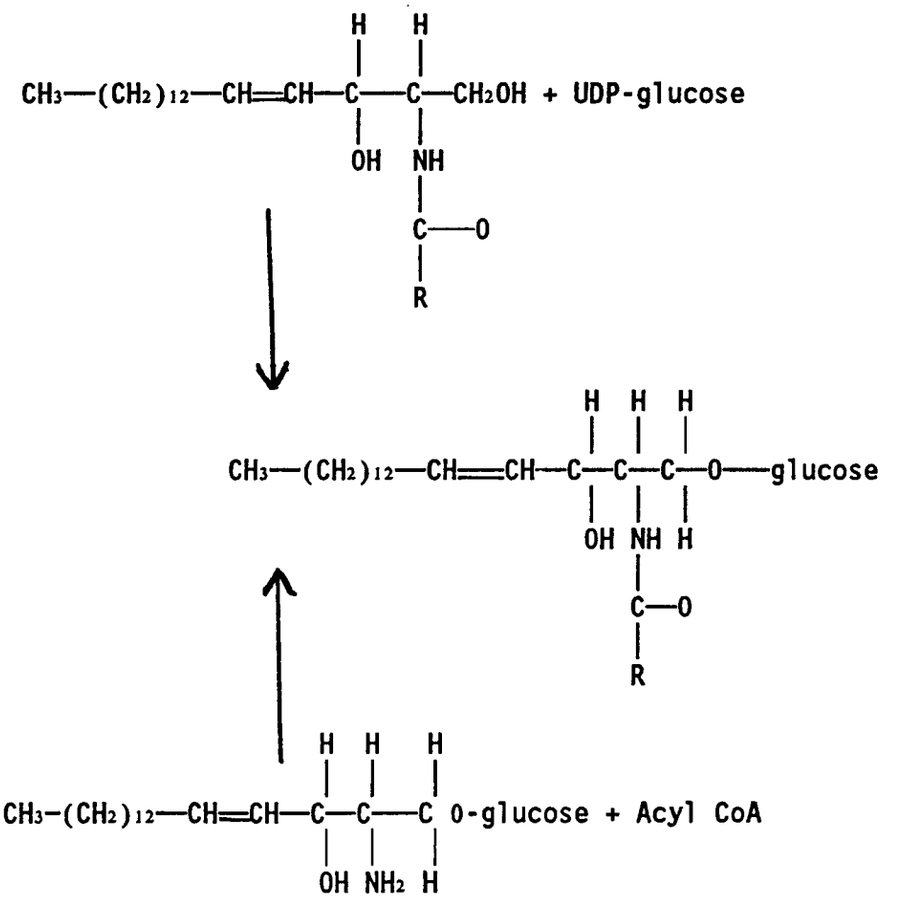

FIGURE 1. Biosynthesis of glucosylceramide by two proposed pathways.

human sources used size exclusion and ion exchange chromatography[32] or affinity chromatography using a natural activator protein[39] as the ligand. Although highly purified enzyme preparations were obtained, these approaches were limited by low yields (<5%)[32] or the small supply of ligand.[39] Methods providing higher yields (≥20%) employed detergent and organic solvent extraction of crude membrane preparations[33] as the initial steps to delipidate and solubilize this tightly membrane associated enzyme.[40] For extraction the bile acid detergents have been used extensively and, in particular, cholate has the distinct advantage of easy removal by dialysis or on desalting columns. Subsequent steps exploited hydrophobic chromatography using short chain alkyl- or aromatic agarose derivatives[33-35] or immobilized phosphatidylserine[17] as ligands. Because of their commercial availability, the former have been used most extensively for obtaining the enzyme that was electrophoretically homogeneous in yield of about 30%.[33] The methods developed by Furbish et al.[33] rely on the elution of the bound enzyme in linear gradients of ethylene glycol from sequential decyl-agarose and octyl-Sepharose™ columns. A stable preparation of this electrophoretically pure enzyme was obtained by cold ethanol precipitation and resolubilization in glycerol and human serum albumin. Since a primary interest of these authors was to develop a preparation that was suitable for administration to affected patients, lectin chromatography was not used due to an unavoidable lectin leakage and the difficulty in its removal.[33] More recently, monoclonal antibodies to acid β-glucosidase have been immobilized and used as affinity ligands for purification of the human enzyme with excellent yields and high purity.[38]

Although electrophoretically homogeneous, the enzyme preparations obtained by hydrophobic chromatography contained human serum albumin,[37] which has the same apparent molecular weight (M_r = 67,000) by SDS-PAGE as the enzyme. Elimination of this and other hydrophobic contaminants was achieved using affinity columns containing immobolized substrate analogs (i.e., *N*-acyl-sphingosyl-β-glucoside derivatives),[36,37] or potent competitive inhibitors (i.e., *N*-alkyl-deoxynojirimycins)[42] (Figure 3). The overall yields of electrophoretically homogeneous enzyme preparations of high specific

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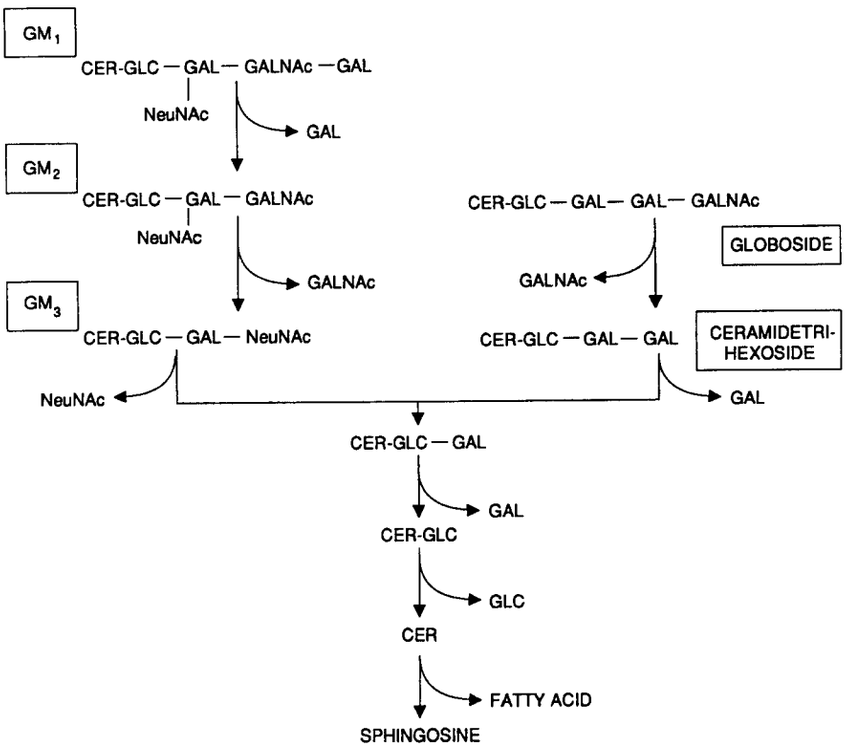

FIGURE 2. Sequential degradation of G_{M1}-ganglioside and globoside by lysosomal enzymes; Glucosylceramide (CER-GLC) is the common intermediate.

activity were about 10 to 25%. Enzyme that was bound tightly to these columns could be eluted with deoxynojirimycin in detergent solutions,[43] δ-gluconolactone in aqueous buffers,[36,37] or high concentrations of ethylene glycol.[37,42] Several human acid β-glucosidase preparations obtained by *N*-alkyl-deoxynojirimycin chromatography have been shown to contain a single N-terminal amino acid sequence and their enzymatic activities were stable for over 1 year at ~20° to 4° in 90% ethylene glycol containing 4 m*M* β-mercaptoethanol.[42]

The final specific activities of the acid β-glucosidase preparations that were electrophoretically homogeneous have varied over a 10-fold range ($\sim 5 \times 10^5$ to 5×10^6 nmol/h/mg protein).[32-35,42] Although this variation might be due to inherent properties of the different enzyme preparations, they also may stem from different compositions of assay mixtures as well as the nature of the substrates used, i.e., glucosylceramide with different sphingosyl or fatty acyl chains as well as 4-methylumbelliferyl-β-D-glucopyranoside (4MU-Glc)[44,45] and, perhaps, the different methods for estimating protein concentrations. Based on active site quantitation using a covalent inhibitor, bromo-[^{3}H]-conduritol B epoxide ([^{3}H]-Br-CBE, Section IV.E), and the assumption that only catalytically active enzyme bound this inhibitor, the catalytic rate constants, k_{cat} of homogeneous human placental acid β-glucosidase for several glucosylceramides and 4-alkyl-umbelliferyl-glucosides varied by 50% about an average of 2350 min^{-1}.[46]

The mutant residual enzyme activity from Gaucher disease tissues has been obtained in a highly purified, but not homogeneous, form.[44,47] The purification properties and yields of acid β-glucosidase from two type 1 Gaucher disease patients were essentially identical to those of the normal enzyme. Most investigators have used partially purified or crude preparations to compare the kinetic or immunologic properties of the mutant and normal enzymes. Because of the extensive heterogeneity of the molecular lesions being delineated at the acid β-glucosidase locus in Gaucher disease patients (see Section V.D), future studies to compare the properties of the normal or mutant acid β-glucosidases will require definition of the precise mutations in particular patients, so that the enzymatic properties could be assigned to specific structural changes.

2. *Physical and Chemical Properties of Acid β-Glucosidase*

Normal human acid β-glucosidase is a homomeric glycoprotein encoded by a single structural locus on chromosome 1.[48-50] Partial[42] and complete[51] amino acid sequences have been determined chemically for the placental enzyme and deduced from the nucleotide sequences of the cDNA isolated from fi-

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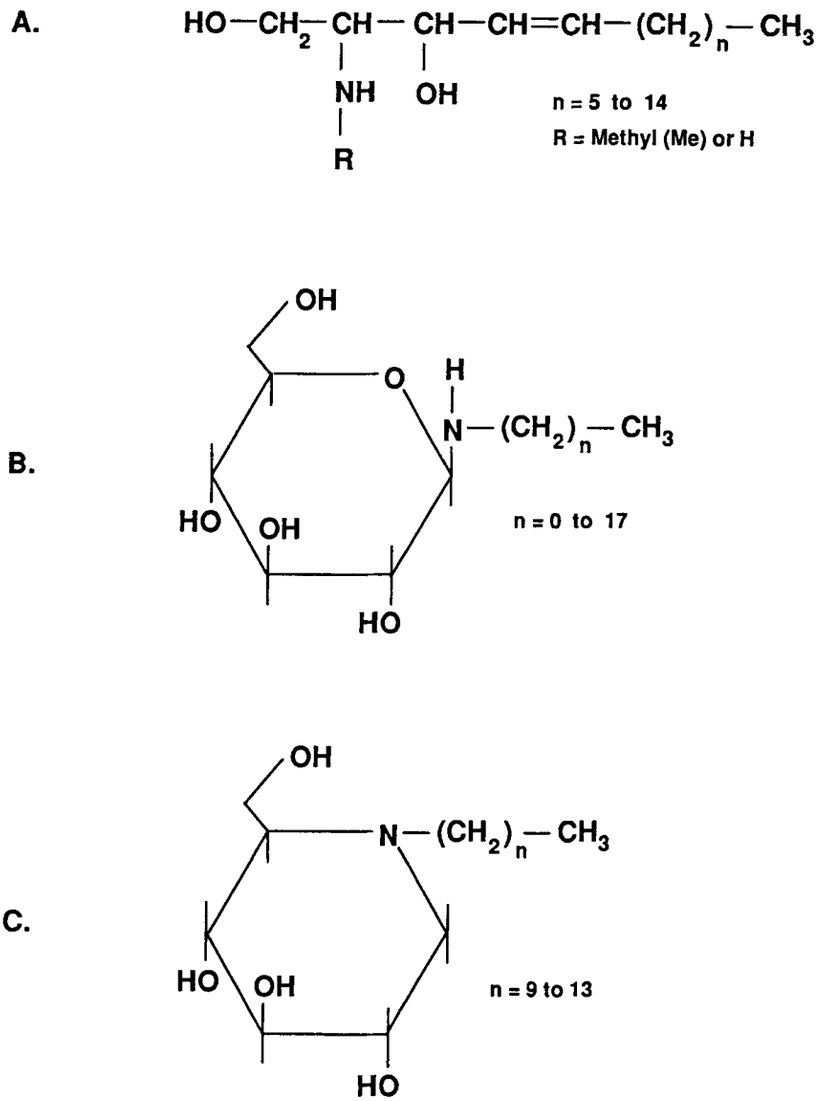

FIGURE 3. Structures of sphingosyl (A), *N*-Alkyl-glucosylamine (B) and *N*-alkyl-deoxynojirimycin derivatives.

broblasts, hepatic and lymphoid[51-55] libraries. Except for the presence of cloning artifacts and mutations casual to Gaucher disease,the amino acid sequences were identical. Similarly, the deduced amino acid sequence from seven overlapping mouse cDNA clones indicated high (88%) identity to the human protein with only one codon deletion in the coding regions.[56] These results and those of immunoelectroblotting studies of several normal tissues indicate that a single polypeptide is translated from acid β-glucosidase specific mRNA in all tissues. This suggests that differences in the *in vitro* kinetic properties of acid β-glucosidases from different tissues cannot be attributed to primary sequence differences.

The mature human polypeptide consists of 497 amino acids and the major physical features of the complete sequence are summarized below and in Figure 4. The calculated molecular weight of this polypeptide is 55,575. About 13% of the residues are basic (lysine, arginine, or histidine) and the calculated pI value is 7.2. This value is consistent with that (pI = 7.3 to 7.8) obtained by isoelectric focusing in granular bed gels[57] for placental enzyme preparations that had been partially deglycosylated to the neutral mannosyl core. Although the protein has about 11% leucine residues and 45% non-polar amino acids, computer calculations using several of the available algorithms and hydrophobicity scales find no stretches of hy-

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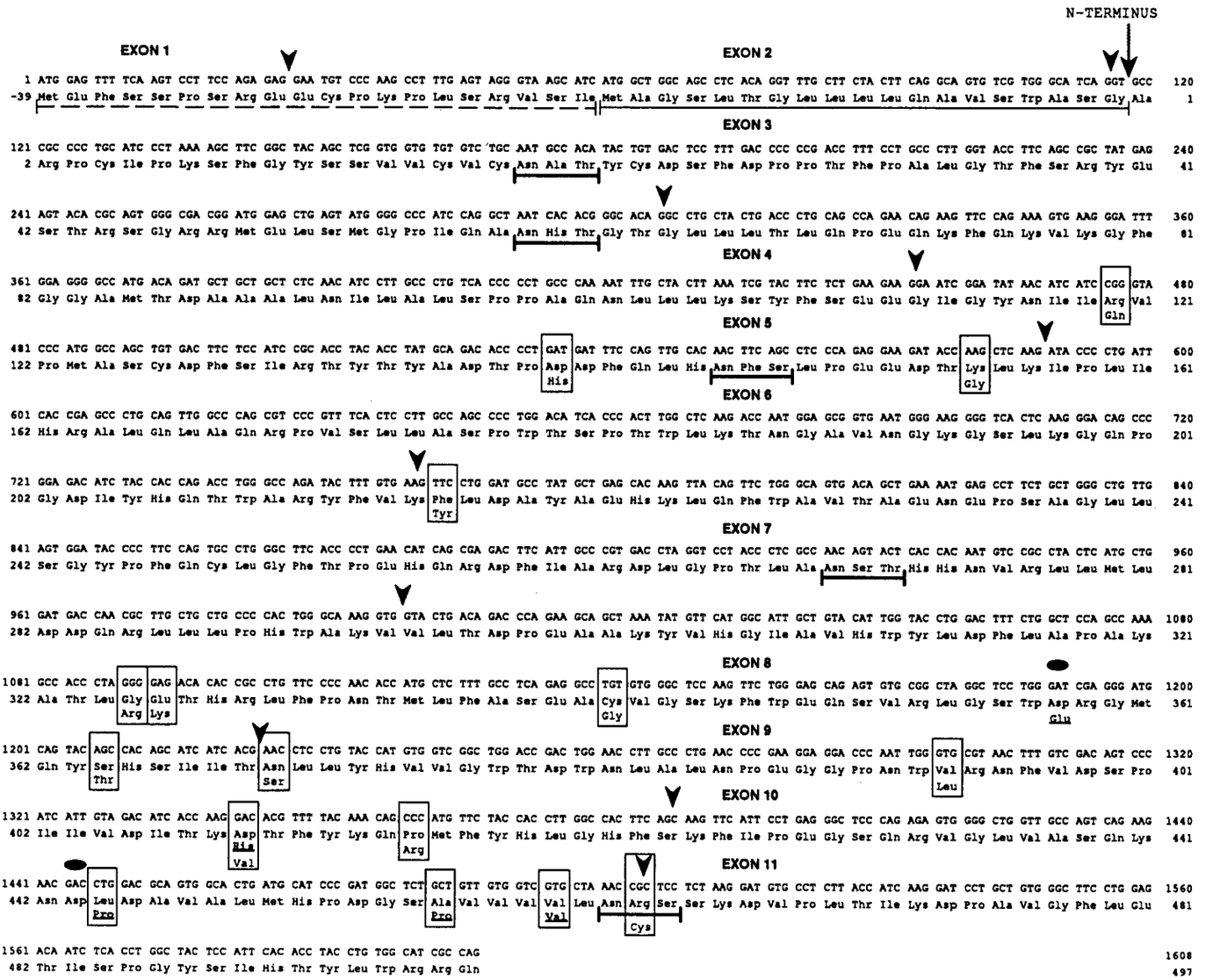

FIGURE 4. Nucleotide sequence of human acid β-glucosidase cDNA and its deduced amino acid sequence. The arrows represent intron/exon boundaries of the human chromosomal gene with the exons indicated above the nucleotide sequences. The ⊢⊣ indicate the five consensus glycosylation sequences. The boxes indicate mutation sites identified in Gaucher disease patients with the amino acid substitution indicated below the normal sequence. The underlined mutations also have been found in the pseudogene. The two indicated aspartates (●) are the proton donor (Asp^{358}, tentative) and the nucleophile (Asp^{443}) for catalysis. *Glu^{358}* represents a random mutation that destroys catalytic activity (see text). The two potential signal sequences are underlined. That following the second ATG has the typical hydrophobic characteristics of other signal sequences.

drophobic amino acids consistent with a transmembrane domain in the mature polypeptide. Also, proteolytic digestion studies of the acid β-glucosidase obtained by *in vitro* translation in the presence of microsomal membranes indicated that human acid β-glucosidase does not have a large cytoplasmic domain.[58] While these findings indicate that acid β-glucosidase is not a transmembrane protein, but rather a peripheral membrane protein, no obvious basis for its tight membrane association is evident from the primary sequence.

Comparisons of the human sequence to that of the murine acid β-glucosidase show that the longest contiguous sequence of different amino acids is four. The remainder of the 57 amino acid differences were scattered throughout the mature proteins with the majority occurring in the N-terminal 50% of the sequence. Furthermore, 100% of the cysteines (7 residues), 91% of the prolines (34 residues), and 89% of glycines (34 residues) were conserved. Interestingly, the three longest sequences of 100% amino acid identity (amino acids 315 to 375, 377 to 406, and 411 to 434) were located in the carboxy-terminal 40% of the sequence.[56] The conservation of these sequences and

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the assignment of an amino acid in the active site[59] to Asp,[443] has led to the proposal that active site functional domains are contained in the carboxy-terminal 50% of the sequence.[56,59]

Comparison of the human and murine sequences also indicated that the presence and placement of the five *N*-glycosylation consensus sequences were strictly preserved, but only those at amino acids 146 to 148 and 462 to 464 were identical. This result also suggests a potential functional role for glycosylation in catalytic activity. The results of recent expression experiments[60] indicate that glycosylation is essential for the catalytic activity of the human enzyme. Takasaki et al.[61] have shown typical bi- and tri-antennary complex N-linked oligosaccharides on the human placental acid β-glucosidase. However, the less common NeuAcα2→3Galβ1 linkages were present, whereas NeuAcα2→6Galβ1 were not.[61] Endoglycosidase F digestion studies of human fibroblast acid β-glucosidase indicated that only four of the potential glycosylation sites are used,[58] but their site occupancy has not been reported. The tissue specific nature of glycosylation and/or site occupancy could provide insight into the potential modulation of acid β-glucosidase activity in different tissues by affecting k_{cat} or the stability of the protein. The former is suggested by the lower k_{cat} of splenic when compared with placental acid β-glucosidase and the latter is suggested by the decreased half-lives of the mutant residual enzyme in some Gaucher disease patient cells[62] (see Section III.C).

3. Acid β-Glucosidase II

Aerts et al.[63] described a second form of acid β-glucosidase in human cells and urine. These investigators used acid β-glucosidase enriched splenic or urine preparations that had been obtained without the use of high salt or organic solvent extraction.[63] With polyclonal and monoclonal antibodies to human acid β-glucosidase about <10% to ~40% of the total enzyme activity could not be precipitated from such preparations. This nonimmunoprecipitable form of acid β-glucosidase was termed glucocerebrosidase II (mol wt ~200,000). The immunoprecipitable form was termed glucocerebrosidase I (mol wt ~60,000). The glucocerebrosidase II from human urine slowly and spontaneously converted at 4°C to glucocerebrosidase I. Also, glucocerebrosidase II reacted with polyclonal antibodies raised to human SAP-2, sphingolipid activator protein 2 (a natural activator of acid β-glucosidase), leading these investigators to conclude that glucocerebrosidase II represented an enzyme/SAP-2 complex. Additional studies to determine the stoichiometry or endogenous lipids in this complex may provide insight into the physiologic state of the acid β-glucosidase/SAP-2 complex.

4. The State of Acid β-Glucosidase in Cells

The native state of acid β-glucosidase in cells and tissues has not been completely defined, since acid β-glucosidase requires detergents or organic solvent extraction for solubilization and endogeneous lipids may be present on the enzyme. This also has bearing on the nature of the acid β-glucosidase in Gaucher disease in which the lipid compositions differ significantly from those of normal tissues.[64] The reported molecular weights of the extracted enzyme, as estimated by sedimentation and molecular exclusion chromatography, have varied from about 60,000 to 450,000.[32-38,42,65,66] Also, molecular weight analyses under nondenaturing conditions have been hampered by the anomalous migration of acid β-glucosidase in polyacrylamide or agarose gels.[35,36] To overcome these difficulties, several investigators have estimated the native molecular weight in tissues by *in situ* radioinactivation of the enzymatic activity.[65-68] The results of two studies[67,68] indicated that the normal enzyme may be dimeric *in situ*, whereas other data[65,66] were consistent with the enzyme being a monomer. Salvayre et al.[65,66] and Choy et al.[67] found a 2- to 3-fold increase in their respective molecular weight estimates for the residual enzyme in Gaucher disease type 1 fibroblasts relative to normal. Consequently, the nature of the acid β-glucosidase in cells remains unresolved, but the physical state of the enzyme in the cell has significant implications for understanding the mechanism of SAP-2 action as well as the effects of the casual mutations in Gaucher disease.

B. Naturally Occurring Protein Activators of Acid β-Glucosidase

In 1971, Ho and O'Brien[27] characterized a heat-stable factor from Gaucher disease spleen that enhanced the hydrolysis of 4MU-Glc by crude preparations of normal acid β-glucosidase. Subsequently, numerous investigators have designed this as "factor," heat stable factor, co-β-glucosidase, Saposin C, and SAP-2 (sphingolipid activator protein 2). SAP-2 will be used in this review. The physiologic importance of SAP-2 was demonstrated by the presence of glycosyl ceramide storage in a patient with normal *in vitro* acid β-glucosidase activity, but with a deficiency of SAP-2 cross-reacting immunological material in several tissues.[69] Recently, a second protein activator of acid β-glucosidase has been described that enhances the hydrolysis of 4MU-Glc and glucosylceramide, *in vitro*. This protein has been designed Saposin A[70] or Protein A.[71] The term Saposin A will be used in this review. Both of these activators derive from a single gene product by extensive proteolytic and glycosidic posttranslational processing (Section III.D).

Several methods have been described for the purification of milligram quantities of SAP-2 to homogeneity from normal and Gaucher disease spleens; all exploit this protein's remarkable thermal stability to heating at 80° to 100°C.[29,31,72,73] This initial step is followed by sequential ion exchange, hydrophobic or concanavalin A chromatography, and/or reversed-phased HPLC. The complete amino acid sequence of the human[72] and guinea pig[72] SAP-2s have been chemically determined, and that from human[71,74-76] and rat[71] have been deduced from their cDNA sequences. The extensive amino acid homologies of SAP-2 from human, rat, and guinea pig are shown in Figure

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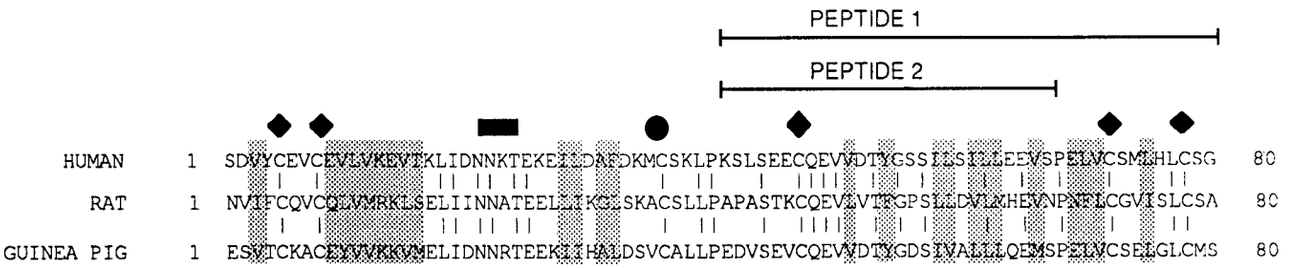

FIGURE 5. Comparison of the amino acid sequences of the human, rat, and guinea pig SAP-2 proteins. Amino acid similarities are shown in shaded regions, while identities are indicated by vertical lines. The shaded diamonds indicate conserved cysteines and the shaded rectangle is a conserved glycosylation site. Peptide 1 and peptide 2 are chemically synthesized and refolded peptides that retain the activating properties of the intact SAP-2. Peptide 1 has greater affinity for and peptide 2 provides higher degrees of activation with acid β-glucosidase.

5. The 80 amino acid (mol wt = 8950 Da) human SAP-2 is a very acidic (pI~4.2) glycoprotein[72] that is consistent with the presence of 20% acidic (Asp and Glu) and 10% basic (Lys and His) amino acids. The protein also contains about 32% non-polar and polar amino acids, as well as six cysteines and two methionines, but no tryptophans (Figure 5). The single N-linked glycosylation consensus is occupied, but only the composition of the guinea pig SAP-2 oligosaccharides is known.[77] The highly acidic nature of SAP-2 as well as the presence of glycosylation probably accounts for the discrepancies in molecular weight determinations and apparently anomalous behavior of this protein on size exclusion supports and native and SDS PAGE systems. The reported molecular weights have varied from 6000 to 22,000.[30,31,44] In addition, several diffuse bands (3 to 4) have been observed on native PAGE that when individually eluted have similar activating effects on acid β-glucosidase.[30] The N-terminal sequences of the proteins in these active bands from purified human SAP-2 are identical[191] suggesting microheterogeneity of glycosylation.

Investigators have suggested that SAP-2 from normal and Gaucher disease sources differed structurally.[31] The recent cloning of its cDNA[71,74-76] from several libraries and the complete chemical determination of the amino acid sequence of SAP-2 from Gaucher disease spleen[72] demonstrated that the amino acid sequences are identical. Radin and co-workers[29,30] and Peters et al.[31] have suggested that the carbohydrate structures on SAP-2s from normal and Gaucher disease spleen differed and that this difference may account for some of the functional differences of the SAP-2s. However, the retention of SAP-2s activating properties after organic solvent extraction[73] cleavage with CNBr[77] and deglycosylation,[77] as well as the complete chemical synthesis and restoration of activity by renaturation,[79] indicate that different contaminants in the various preparations from normal and Gaucher disease tissues might account for these observations. These latter studies[79] localized the activating function to residues 41 to 80 of the mature SAP-2. As shown in Figure 5, Peptide 1 (amino acids 41 to 80) had greater affinity for acid β-glucosidase but maximal activation was achieved with Peptide 2 (amino acids 41 to 68). The structure of SAP-2 in solution has been investigated by NMR spectrometry that suggested the presence of a rigid hydrophobic pocket as a component of SAP-2 folding.[80]

The recently described acid β-glucosidase activator, Saposin A, has been purified to homogeneity from human spleen by heat treatment and HPLC.[70] The complete amino acid sequence has been chemically determined[70] and predicted from the cDNA sequence.[71,75] Similar to SAP-2, Saposin A is small (M_r = 9148), very acidic (calculated pI = 4.14), and has six cysteine residues that align with those in SAP-2 (Figure 6). However, Saposin A has two *N*-glycosylation consensus sequences and both are occupied.[70] By comparison to a rat cDNA, which encodes the homologous sequence, the human and murine proteins are essentially identical (89% amino acid identity). More extensive comparisons are discussed in Section V.E.

IV. BIOSYNTHESIS AND PROCESSING OF ACID β-GLUCOSIDASE AND ITS NATURAL PROTEIN ACTIVATORS

A. Posttranslational Processing of Normal Acid β-Glucosidase

Biosynthesis of normal acid β-glucosidase has been investigated in cultured porcine kidney cells and human skin fibroblasts.[58,62,81,82] Such studies were undertaken, in part, to determine the origin of three molecular weight forms of acid β-glucosidase observed by immunoelectroblotting of human skin fibroblast[83-86] and brain extracts.[83] Total removal of N-linked oligosaccharides from acid β-glucosidase from human fibroblasts with *N*-Glycanase™ resulted in the detection of a single immunoreactive form (M_r = 56,000) of acid β-glucosidase.[85] These finding demonstrated that the three forms of the enzyme in extracts of these cells were derived from the same polypeptide chain by differential posttranslational oligosaccharide remodeling.[85] Pulse-chase processing techniques together with endoglycosidase digestion were used to evaluate the temporal

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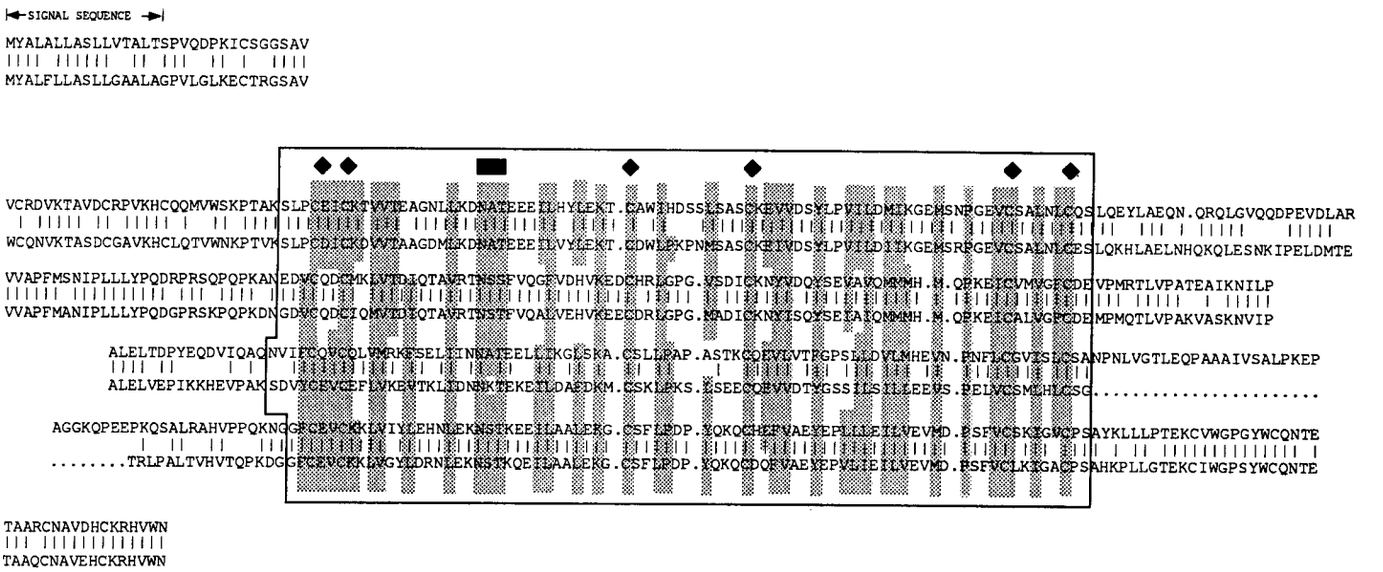

FIGURE 6. Similarity and conservation of functional and theoretical peptide coding regions of rat Sertoli cell sulfated glycoprotein-1 (SGP-1) (top sequences) and human proactivator (bottom sequences) corresponding to Saposin A, SAP-1, SAP-2, and protein C. The signal sequence for SGP-1 was predicted from the chemically determined N-terminus (Collard et al. 1988). That for the human proactivator was predicted by computer analyses. The boxed area indicates the four regions of the mature peptides in the human proactivator sequence. The solid diamonds indicate aligned cysteine residues, and the solid rectangle indicates conserved *N*-glycosylation sites. Shaded areas indicated conserved similarities of amino acids with like properties.

relationships and the nature of the oligosaccharide remodeling that accounted for the steady-state forms of the enzyme.

Using ^{35}S-methionine metabolic labeling, the first detectible forms of the human[58,62,82] and porcine[58] enzymes were glycosylated and contained high mannosyl chains,[58,62] which indicated residence in the endoplasmic reticulum. Over 1 to 24 h, these initial forms were transformed quantitatively into a higher molecular weight form that was not completely sensitive to endoglycosidase H treatment.[58,62] This transformation was due to the remodeling of the oligosaccharide chains to complex types within the Golgi apparatus as demonstrated by the abolition of the increase in molecular weight by treatment with neuraminidase. This high molecular weight form was converted over about 72 h to a final glycosylated form with lower apparent molecular weight (M_r = 59,000) than the initial form with M_r = 63,000. Timed endoglycosidase H digestion of the procine enzyme metabolically labeled for 5 min (i.e., only enzyme containing high mannosyl oligosaccharides in the endoplasmic reticulum would be evaluated) indicated that four of the potential five *N*-glycosylation consensus sequences were glycosylated. Using *in vitro* cell-free translation in the presence of dog pancreatic microsomal membranes, Erickson et al.[58] showed the typical proteolytic clipping of a signal sequence coincident with transport of the synthesizing polypeptide chain across the endoplasmic reticulum. Deglycosylation experiments in conjunction with metabolic labeling demonstrated that the transport of the enzyme from the Golgi apparatus to the lysosomes was not associated with detectible proteolytic processing[62] as had been observed with several other lysosomal hydrolases.[87-89] Based on similar studies, the rate of transport of acid β-glucosidase from the endoplasmic reticulum to the Golgi apparatus was estimated at 3 h, which was long compared with that for other lysosomal enzymes.[88] The half-life of acid β-glucosidase in cultured skin fibroblasts was estimated to be about 60 h, which is relatively short for lysosomal enzymes.[62]

B. Trafficking of Acid β-Glucosidase to the Lysosome

The presence of an exposed mannose-6-phosphate on the oligosaccharide side chains of many glycoproteins is now accepted as important for their targeting to the lysosome in some tissues via the cation independent receptor.[90-93] The lack of such residues due to the genetic deficiency of a *N*-acetylglucosamine-1-phosphotransferase[92,93] in I-Cell disease leads to the secretion of the majority of soluble lysosomal hydrolases into the media of patient fibroblasts or plasma. However, acid β-glucosidase and acid phosphatase are retained in I-Cell fibroblasts at normal levels. Consequently, it seems likely that these enzymes that become membrane associated are trafficked and sorted to the lysosome by signals other than mannose-6-phosphate.[94] To date, the signals for this alternative targeting are unknown. However, the mechanism by which acid phos-

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phatase, a similar membrane-associated lysosomal protein, is trafficked to the plasma membrane and then reinternalized via carboxy-terminal signal[94] may apply to acid β-glucosidase.

The potential role of oligosaccharides in the targeting of acid β-glucosidase to the lysosome was evaluated in the human monoblast line U937 by inhibiting selected steps in oligosaccharide remodeling.[95] Swainsonine inhibition of Golgi α-mannosidase II results in hybrid oligosaccharide side chains with one unprocessed high-mannose branch and one branch with *N*-acetyl-glucosamine and additional terminal sugars.[96] Deoxymannojirimycin inhibition of Golgi α-mannosidase IA/B results in presence of only high-mannose oligosaccharides.[97] Using blockades of the complex oligosacchardie processing pathway by these inhibitors, enhanced activity of acid β-glucosidase was observed in the absence of effects on soluble lysosomal hydrolyzed, e.g., β-hexosaminidase, α-galactosidase, α-fucosidase, or β-galactosidase activities. No additional increase in acid β-glucosidase activity was observed in the presence of leupeptin, a lysosomal-directed thiol protease inhibitor. In addition, Percoll® gradient fractionation indicated that treatment of U937 cells with either of the glycosidase inhibitors led to an accumulation of nearly all of the acid β-glucosidase in a fraction that contained galactosyltransferase activity, i.e., a presumably Golgi-enriched fraction. The distribution of β-hexosaminidase was not altered. These findings were interpreted to suggest that the presence of complex type oligosaccharide side chains were "essential for efficient routing of the enzyme to the lysosome."[95] However, a more precise interpretation will require metabolic labeling studies with preparations of Golgi that are free of "light" lysosomes and an accurate determination of the half-life of acid β-glucosidase in the cells.

C. Posttranslational Processing of Acid β-Glucosidase in Cells from Gaucher Disease Patients

Ginns et al.[83] suggested a differential processing of the mutant residual acid β-glucosidase in fibroblasts from patients with Gaucher disease variants. By immunoblotting the three (M_r = 63,000 and 56,000) steady-state forms of normal acid β-glucosidase were present in cultured skin fibroblasts from type 1 patients. In comparison, only the 63,000 form was detected in cultured skin fibroblasts from types 2 and 3 patients with rabbit anti-human acid β-glucosidase antiserum. No immunoreactive material was observed in type 2 fibroblast extracts with a monoclonal antibody to acid β-glucosidase that did detect a M_r~63,000 form in type 3 sources.[83] Using similar techniques, Beutler et al.[98] observed that only a single form of acid β-glucosidase immunoreactive material was detectible in fibroblasts extracts from normal or any type of Gaucher disease patient. These investigators concluded that this technique was not useful for the diagnostic discrimination between the variants of Gaucher disease. A similar conclusion was reached by Fabbro et al.[85] based on immunoelectroblotting of fibroblast extracts from a very ethnically heterogeneous population of Gaucher disease patients. Using polyclonal or monoclonal antibodies, the patterns of molecular weight forms of immunoreactive material detect in most Ashkenazi Jewish Gaucher disease type 1 and some non-Jewish type 1 samples were normal and each of the immunoreactive form (M_r = 67,61 to 64 and 58,000) bound the specific covalent active site-directed inhibitor [^{3}H]-Br-CBE. In comparison, the majority of fibroblasts extracts from non-Jewish type 1 and types 2 and 3 patients had one or two forms of immunoreactive material corresponding in migration to normally present bands. Deglycosylation studies indicated the presence of normal molecular weight of the mature acid β-glucosidase polypeptide chains in all variants of Gaucher disease.[85]

Using metabolic labeling, normal processing patterns, and nearly normal time courses for processing of acid β-glucosidase were determined in Gaucher disease types 1[62,82] and 3 fibroblasts.[82] In comparison, in the type 2 fibroblast line, HMCR GM877, acid β-glucosidase was synthesized as a M_r = 60 to 63,000 initial form, was not further processed, and was completely degraded in <24 h. Bergmann and Grabowski[62] found the normal processing sequence in most Ashkenazi Jewish type 1 patients and in those non-Jewish patients type 1 previously determined to have normal patterns of enzyme forms by immunoblotting.[62] However, the truncated processing pattern of acid β-glocisidase in the Gaucher disease type 2 GM 877 cells was not specific for either the variant type nor the ethnic group. For example, truncated processing indistinguishable from GM877 was found in Ashkenazi Jewish and non-Jewish type 1 patients. Furthermore, the initial 64,000 form of acid β-glucosidase was partially processed to an unstable higher molecular weight form (M_r = 69,000) in an "atypical" type 2 cell line GM2627. These results indicated an extensive genetic heterogeneity within and among the Gaucher disease variants (see Section V.D). Although the major outlines of the biosynthesis of mutant acid β-glucosidases in Gaucher disease cultured fibroblasts have been defined, these studies do not provide a hypothesis to account for the presence or absence of neuronopathic involvement.

A simple hypothesis to explain the presence of neuronopathic disease would be a significantly lower effective level of acid β-glucosidase activity in the lysosome of particular cells compared with that in nonneuronopathic Gaucher disease. Ultrastructural localization studies[99] of acid β-glucosidase indicated the presence of colloidal gold coupled anti-acid β-glucosidase antibody at the lysosome in cultured fibroblasts from normal individuals and three of four type 1 Gaucher disease patients. These results were interpreted to suggest a dificiency of acid β-glucosidase antigen at the lysosome in the neuronopathic variants.[99] However, the fact that acid β-glucosidase arrives at the lysosome in cultured fibroblasts from type 1, 2, and 3 patients was demonstrated by the increased level of the residual activity in cells grown in the presence of leupeptin.[100]

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D. Biosynthesis and Processing of SAP-2

Fujibayashi and Wenger[101] used rabbit anti-human SAP-2 antibodies in metabolic labeling studies to demonstrate that SAP-2 in human skin fibroblasts had extensive proteolytic and glycosidic processing. In chase experiments following a short pulse (15 min), the major product was a 68,000 Da glycosylated species that over 1 h was converted to a 73 kDa form. Subsequently, the total amount of these two species decreased coincident with the appearance of diffuse species with M_r centered at about 12,000 and 9000. In addition, a SAP-2 specific M_r = 50,000 species remained relatively constant throughout this period. After 1 h, the 73, 68, and 50 kDa species decreased proportionately and were absent by about 2 to 3 h of chase. By 5 h of chase the 12 kDa species had nearly disappeared, while the 9 kDa was predominant. Endoglycosidase F digestion studies of [^{35}S]-methionine-labeled SAP-2 immunoprecipitates demonstrated that the 73 and 68 kDA or the 12 and 9 kDa species were glycosylated species with polypeptides of M_r = 50,000 or 7,600, respectively. It is notable that very similar results were reported for the biosynthesis of SAP-1,[102] which would be expected now that SAP-2 and SAP-1 have been demonstrated to be encoded by the same gene and mRNA.[71,74-76] However, neither the sequence or events involved in proteolytic processing of the "proactivator" precursor nor their potential tissue specificity have been elucidated. The finding of a highly homologous precursor produced in rat Sertoli cells (SGP-1),[71,103] which is not proteolytically processed and is secreted into the culture media suggests that either tissue specificity or particular sequence differences could account for these differences.[71]

V. ENZYMOLOGICAL ASPECTS OF ACID β-GLUCOSIDASE

A. Assay Systems

Kinetic studies of acid β-glucosidase have been complicated by the fact that the enzyme is membrane associated, requires detergents and/or delipidation for solubilization, and specific amphiphiles for reconstitution of enzymatic activity. In a fully delipidated, soluble form the enyme has essentially no catalytic activity. The enzyme's natural substrates, glucosylceramides, have been identified by their accumulation in the tissues of patients with the Gaucher disease variants.[21] Small quantities of glucosylsphingosines have been found in Gaucher disease tissues, suggesting that these deacylated analogs of glucosylceramides also may be natural substrates for acid β-glucosidase.[21,64] Glucosylceramides are insoluble in aqueous media and require a lipoidal environment for dispersion. In comparison, a widely used synthetic substrate, 4MU-Glc, has greater but limited solubility in aqueous dispersions. Consequently, the assays for acid β-glucosidase are conducted in heterogeneous dispersions whose varying physical compositions and phase states may influence the physical state and activity of the enzyme as well as its interactions with effector molecules. Despite these limitations, there has been a remarkable consistency in the results from various laboratories that employed (1) micellar and mixed micellar systems with combinations of polyoxyethylene ethers (e.g., Triton® X-100); bile salts (e.g., sodium taurocholate); and/or fatty acids (e.g., oleic acid), or (2) negatively charged phospholipids or glycosphingolipids dispersed by sonication. The difficulties with these assay systems, particularly those using negatively charged lipid dispersions, is the poor definition of their physical state that may be dependent on the dispersion procedure and the intrinsic properties of the lipids, for example, when sonicated in aqueous medium dispersions of pure phosphatidylserine form unstable liposomes.[104]

B. Detergents and Bile Salts

In the absence of detergents or negatively charged lipids, delipidated human,[40,41,105,106] bovine,[43] and murine[105] acid β-glucosidases have little hydrolytic activity toward 4MU-Glc or glucosylceramide substrates. In addition, kinetic values (i.e., K_m and V_{max}) obtained in the absence of dispersants cannot be interpreted due to the aggregated state of the enzyme and glucosylceramide substrates.[41] To reactivate the enzymes, nonionic polyoxyethylene ether detergents (e.g., Triton® X-100) have been added to the assay mixtures resulting in enzymatic activity toward both 4MU-Glc and glucosylceramides substrates. Similar "activating" effects have been observed by the addition of negatively charged bile salts alone to assay mixtures, but large amounts of these compounds are required for initial effects[40,41,43] and higher levels of activation are ultimately achieved. This finding probably relates to the dispersion of water-insoluble substrates as well as the hydrophobic enzyme, thereby permitting their reaction. The bile salts also have a specific interaction with the enzyme that is mediated via their net negative charge[107] and is related to their critical micellar concentrations (CMCs). Using crude enzyme preparations from leukocytes or purified enzyme similar dependencies were obtained with the 4MU-Glc[21,108] and glucosylceramide[45] substrates. Using glucosylceramide or 4MU-Glc substrates and assuming Michaelis-Menten kinetics, dissociation constant for taurocholate (K_A) of about 4 to 5 m*M* was assigned to taurocholate.[45] Since this value is similar to the CMC for this bile salt, the result suggested that monomeric bile salts may interact with acid β-glucosidase at a binding site. Glew et al.[107] have indicated a marked increase in thermal lability of the human enzyme in the presence of bile salts, suggesting their direct interaction with the enzyme. A similar dependence of acid β-glucosidase activation on CMC was found in Triton® X-100 free systems using homologous series of alkyl-sulfates (e.g., SDS) and sulfonates[192] whose potency of activation was related to their alkyl chain length with either glucosylceramide or 4MU-Glc substrates. However, at high

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concentrations (i.e., >CMC) decreasing hydrolytic rates were observed with either substrate due to enzyme denaturation.

The polyethylene ethers, bile salts, and other synthetic negatively charged lipids will continue to have roles in the enzymology of acid β-glucosidases from normal and Gaucher disease tissues (see below) since numerous assay procedures for the diagnosis of this disease and its carrier state are based on their use.[106-117] These compounds are available in large quantities in pure form and are not subject to oxidation and batch variations. However, they are not of physiologic importance and their *in vitro* use obscures the effects of many components for *in vivo* acid β-glucosidase activity (e.g., SAP-2).

C. Negatively Charged Phospholipids And Glycosphingolipids

Because of the *in vivo* membrane association of acid β-glucosidase, investigators reasoned that specific membrane-derived lipids might be required for enzymatic activity. Based on the potent activating effects of taurocholate, Ho and O'Brien[27] suggested that the negatively charged phospholipids might be the natural "activators" of this enzyme. Following thorough delipidation of crude enzyme preparations, Dale et al.[41] and Berent and Radin[29] demonstrated that acid β-glucosidase activity toward 4MU-Glc was enhanced by negatively charged phospholipids in the following series: Phosphatidylserine > phosphatidylinositol > phosphatidic acid, whereas phosphatidyl-ethanolamine or -choline had no effect on enzymatic activity. These results have been reproduced by numerous investigators in human, bovine, and murine sources,[40,42,43,45,106] albeit with some variation in the composition of the assay mixtures and in the degree of activation. Using crude or purified human acid β-glucosidase, detergent-free assay systems were developed based on glucosylceramide incorporation into unilamellar liposomes. In these artificial membrane systems, optimal activity also required the presence of negatively charged phospholipids to which acid β-glucosidase directly binds.[118] Sarmientos et al.[119] indicated that maximal activity was obtained with about 19 mol% of phosphatidylserine in liposomes, a content of the same order of magnitude as found in lysosomal membranes.[105]

In a series of publications, Glew and co-workers delineated some structure/activity relationships between acid β-glucosidase and negatively charged phospholipids or glycosphingolipids.[105,124-129] These studies were conducted with delipidated, partially purified acid β-glucosidase preparations and 4MU-Glc as substrate in the absence of detergents. Using a series of phosphatidylglycerols with homogeneous fatty acid chains in the *sn*-1 and *sn*-2 positions, maximal activation of acid β-glucosidase activity was found with di-C_{12} saturated derivatives. Decreasing activation effects were found with increasing fatty acid chain length up to di-C_{18} saturated derivatives. In comparison, the di-$C_{18:1}$ and di-$C_{18:2}$ unsaturated derivatives were 3- to 5-fold more potent than the saturated analogs.[83] These unsaturated di-C_{18} phosphatidylglycerols were more potent activators than bovine brain phosphatidylserine in the absence of SAP-2 (see below) but less potent in its presence. Similar results were obtained with a series of *N*-acyl-phosphatidylethanolamines.[106] Since acylation of the parent compound confers a net negative charge on the products, the fact that activation was found only with the acylated products demonstrated the necessity of the charge. The nature of the charged group was not critical, since mono- and di-acyl (C_7 to C_{12}) glycerolsulfates had produced similar levels of activation.[125] In the presence or absence of SAP-2 and within a factor of about two, the *N*-acylated derivatives with acyl chains of 2 to 24 carbons produced very similar degrees of activation. In comparison, *N*-acylation of phosphatidylserine obliterated the potent activation effects of the parent compound.[126] Although the chain length of the *N*-acyl group was not specific, acylation would be expected to increase the net negative charge on the polar head group, analogous to the potent activator, phosphatidic acid.[41,119] Consequently, the *N*-acylation could change the aggregation state of the parent compound that has been shown to affect the ability of negatively charged lipids to activate the enzyme.[124]

The necessity of a negative charge in the polar head group of lipids for activation suggested that charged membrane glycosphingolipids might also be activators of acid β-glycosidase. Indeed, the gangliosides, G_{M1}, G_{M2}, G_{M3}, G_{D1}, and G_{T2}, have been shown to weakly activate the murine and human enzymes, whereas asialo-G_{M1} and G_{M2} did not.[40,119,121] Intriguingly, the presence of *bis*-(monoacylglyceryl)phosphate in liver lysosomal membranes and the potential tissue specificity of lysosomal lipid compositions suggest that the lipid milieu of acid β-glucosidase in the intact lysosome may play a role in modulating its activity in different tissues.[121] Characterization of the lipids associated with acid β-glucosidase from different tissues could provide an interesting insight into the enzyme's *in vivo* functional hydrophobic environment.

D. Naturally Occurring Protein Activators

Two small glycoproteins, SAP-2 and Saposin A, have been shown to activate acid β-glucosidase *in vitro* in the presence of negatively charged phospholipids. Although SAP-2 activated the *in vitro* activity of sphingomyelinase and galactosyl ceramide: β-galactosidase, storage of the respective substrates for these enzymes were not reported in the patient with SAP-2 deficiency.[69] Patients with Saposin A deficiency have not yet been described. SAP-2 has been studied extensively by many investigators[27,29,45,78] since its fortuitous discovery by Ho and O'Brien.[27] Unlike SAP-1 that binds to various substrates and might function as a transfer protein,[127] SAP-2 interacts directly with the enzyme[27,29] and does not interact with glucosylcer-

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amide under equilibrium or nonequilibrium conditions.[29] Consequently, SAP-2 enhances the hydrolysis of both glucosylceramide and 4MU-Glc substrates[29,44,45] in the presence of a variety of negatively charged lipids. Polyoxyethylene ether detergents or bile salts at relatively low concentrations interfere with the activating effects of SAP-2[27,29] as do high concentrations of negatively charged phospholipids.[29] Berent and Radin[29] have suggested that at low concentrations phosphatidylserine interacts with an anionic site on acid β-glucosidase, whereas at high concentrations, it decreases the activation effects of SAP-2 by competing for this site on the enzyme. Although thoroughly delipidated SAP-2 has little or no activating effects on acid β-glucosidase, Glew and co-workers[78,128,129] have indicated that the lowering of the enzyme's K_M for the 4MU-Glc substrate in the absence of additives constitutes evidence for SAP-2's interaction with the enzyme. The activating effects of SAP-2 on the hydrolysis of substrates by acid β-glucosidase have absolute requirements for negatively charged phospholipids or glycosphingolipids.[29,78] These effects are thought to be mediated by conformational changes in acid β-glucosidase so that the interaction of phosphatidylserine conforms the enzyme into a "poised" form for the interaction of SAP-2.[29] Although the mechanism of SAP-2 action is not known precisely. Prence et al.[78] found that the sedimentation rate of delipidated murine acid β-glucosidase in the presence of phosphatidylserine and a cyanogen bromide cleavage fragment of human SAP-2 was consistent with the formation of a dimeric enzyme/SAP-2/lipid active complex.[78]

Similar kinetic results were reported for the effects of Saposin A.[70] The only difference between the effects of Saposin A and SAP-2 was that the former maintained its activating effects in the presence of Triton® X-100. As with Sap-2, Saposin A also increased the hydrolysis of galactosyl ceramide by its β-galactosidase. In contrast to the *in vitro* effects of SAP-2, Saposin A inhibited sphingomyelinase activity by 30%. The fact that both SAP-2 and Saposin A derive from the same peptide precursor by proteolytic processing suggests the physiologic involvement of these activators in glycosphingolipid degradation. However, the fact that the single patient with SAP-2 deficiency had a glucosylceramide storage disease indicated that the physiologic action of Saposin A alone was insufficient to maintain normal glycosphingolipid flux in cells.

E. Properties of the Active Site

1. Substrate Specificity

The natural substrates for acid β-glucosidase are a mixture of *N*-acyl-sphingosyl-1-*O*-β-D-glucosides, glucosylceramides, with differing fatty acid acyl and sphingosyl moieties depending upon the tissue source.[22,130] The enzyme is specific for D-glucose since the L-glucosyl derivative is not hydrolyzed.[131] A minor product, β-glucosylsphingosine, the deacylated analog of glucosylceramide,[21] has been identified in tissues from severely affected Gaucher disease patients. The length of the sphingosyl moiety of these substrates varies between about 18 and 22 carbons, with the C_{18}-sphingosine being in the greatest abundance.[21,64] For a variety of assay systems, natural glucosylceramides isolated from Gaucher disease tissues or synthetic glucosylceramides containing radiolabeled glucose[1,2] or radiolabeled or fluorescent[132] fatty acids are excellent substrates for crude or pure preparations of acid β-glucosidase. Glucosylsphingosine and its *N*-alkylated derivatives, bearing a positive change on the C2 nitrogen of spingosine, are hydrolyzed at 25 to 100-fold lesser rates than glucosylceramides.[44,119,133,134] The influence of the fatty acid acyl chain length (1 to 18 carbons) on substrate hydrolysis was investigated with a series of synthetic glucosylceramides.[44] Using a mixed micellar assay system with Triton® X-100 and taurocholate, maximal hydrolytic rates were achieved, with glucosylceramides containing fatty acids of 8 to 18 carbon atoms. These rates were about 10-fold greater than those found with the formyl derivative, whereas the stearyl derivative was hydrolyzed at about 60% of maximal rates. The length of the fatty acid acyl chain had small effects on the apparent K_M values.[44,119] Sarmientos et al.[119] examined the effects of the configuration and substitutent groups of the polar head groups of the ceramide moiety on hydrolytic rates. They found that the D-glucosyl-D-*erythro*-ceramides had somewhat lower $K_M$app values and higher V_{max} values than the corresponding D-glucosyl-L-*threo* derivatives. The presence of the C4-C5 *trans* double bond in the sphingosyl moiety of glucosylceramide has been shown in micellar[44,135] and liposomal[119] systems to reduce apparent affinity and hydrolysis by acid β-glucosidase. The influence of the length and composition of the sphingosyl chain on kinetic parameters has been investigated by inhibition studies[136] (see below).

The synthetic substrate, 4-methyl-umbelliferyl-β-D-glucopyranoside (4MU-Glc), provides excellent estimates of acid β-glucosidase activity, is easy to use, is inexpensive, and is commercially available in large quantities. From the extensive literature on this substrate with innumerable combinations of detergents, phospholipids, and fatty acids and in crude tissue extracts or purified enyme preparations, the apparent $K_M$app for 4MU-Glc is about 1.5 to 4 m*M*, about 100-fold greater than that for glucosylceramides. In addition, $K_M$app was nearly constant over a pH range of 4.2 to 7.25, while V_{max}/K_M varied by over an order of magnitude.[137] Taken together the above results indicate that the K_M for this substrate probably represents K_S, the thermodynamic dissociation constant. Increasing the length of the 4-alkyl group of this substrate from 7 to 11 carbons decreased $K_M$app by an order of magnitude without a change in V_{max} or kcat[43,46] These longer chain 4-alkyl-U-Glc

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derivatives are excellent substrates and provide interesting probes of the acid β-glucosidase active site. Using sugar-substituted 4MU derivatives, homogeneous acid β-glucosidase has little hydrolytic activity toward non-β-glucose compounds, except possibly the β-xylose derivative.

Using glucosylceramide or 4MU-Glc as substrates, the pH optimum for hydrolysis has varied somewhat with the composition of the assay mixtures, i.e., micellar, phospholipid dispersions, or liposomal. For all studies the profile of pH variation of hydrolytic rates was broad, about 2 pH units and rather shallow, i.e., the maximum variation in rates was about 10-fold. Using purified human acid β-glucosidase in a mixed micellar system and maintaining constant ionic strength as well as enzyme stability at all pH values, the V_{max} profiles for the glucosylceramide or 4MU-Glc substrates conformed to a diprotic model with a monoprotonated active form.[137] The ionizable groups had pK_a values of about 4.7 and 6.7 with a V_{max} optimum at pH = 5.75. Murine, bovine, and human acid β-glucosidase in various stages of purification conform to a similar model with pH optima within ±0.5 pH units and resultant shifts in estimated pK_a values for the ionizable groups. For studies that were conducted in the presence of negatively charged phospholipids and SAP-2, more acidic optima were obtained, whereas those in detergent-based systems were more basic.[78] The acidic pK_a = 4.7 was consistent with the identification of Asp^{443} as a catalytically necessary nucleophile[59] (see below). The identify of the pK_a = 6.7 groups is not known but recent mutagensis data suggest that it may correspond to an Asp^{358} (Figure 4) residue with a shifted ionization constant and that functions as an acid for proton donation during catalysis.[139]

2. *Inhibitor Studies*

Using their results[44,45,136-139] and previous work[140,141] of substrate specificity and inhibitor interactions, Grabowski and coworkers have proposed a kinetic model of the active site of acid β-glucosidase that includes three binding sites for the glycon head group, the sphingosyl moiety, and the fatty acid acyl chain of glycosyl ceramide (Figure 7). Interaction at the glycon binding site requires a specific bonding with each hydroxyl group on glucose, while the sphingosyl and fatty acid acyl binding sites are thought to accommodate hydrophobic chains up to about 18 carbon atoms in length. These lattter sites have been proposed to be composed of multiple subsites that accommodate single methylene groups on the alkyl chains of substrates and inhibitors.[43-45] This model is similar to those proposed for the binding of globotriaosylceramide to SAP-1[142] and phospholipids to phospholipid transfer protein.[143] Although additional supporting structural data are required for these models, they may serve as prototypes for binding sites on lipid transfer proteins and partial or complete active sites of glycosphingolipid hydrolases.

a. SUBSTRATE AND SPHINGOSYL ANALOG INHIBITORS

Erickson and Radin[140] showed that a series of positively charged secondary 2-amino glucosylceramide analogs, the *N*-alkyl-glucosylsphingosines, were highly potent inhibitors of acid β-glucosidase. Furthermore, the introduction of a basic group in place of the corresponding non-basic 2-amide of ceramide increased the apparent affinity for acid β-glucosidase by about 100-fold. Maximal inhibitory potency was found with the *N*-hexyl to *N*-dodecyl derivatives ($K_i \sim 500$ n*M*), whereas the *N*-octadecyl-glucosylsphingosine was less potent. This maximal inhibitory potency of *N*-hexyl-glucosylsphingosine was observed at pH ~7 to 8, a pH at which the majority of the enzyme would be present as an inactive diprotonated form. Since the pH profile for the inhibitory potency plateaued at pH values from 6 to 8, while the activity was decreasing dramatically, it is likely that this extremely potent cationic inhibitor was interacting with an important anionic residue on the enzyme that was not directly involved in catalysis. Several investigators have reported the potent noncompetitive inhibition of acid β-glucosidase by glucosylsphingosine (K_i = 20 μ*M*) and its *N*-alkyl derivatives[44,45,119,134] in a variety of assay systems using either glucosylceramide or 4MU-Glc as substrates. This was postulated to result from the presence of sites on acid β-glucosidase for the preferential binding of substrate or this inhibitor.[44,45,124] Glucosylsphingosine also was a substrate, albeit a poor one, and Vaccaro et al.[134] showed co-purification in a constant ratio of the glucosylceramide and glucosylsphingosine hydrolytic activities. However, the two activities differed in their lipid and detergent requirements and, possibly, their thermal stabilities.[134] The pH optima of the two activities also differed with the glucosylsphingosine hydrolytic activity being more acidic than that for glucosylceramide,[134] both activities conformed to a diprotic model. These results suggested that the residues that serve as the proton donors for *O*-glucosidic cleavage for these two substrates could be different. The following findings suggest that the nucleophile for catalysis of either substrate is the same:(1) CBE (see below) inhibits glucosylceramide and glucosylsphingosine hydrolysis by pure human placental acid β-glucosidase,[191] (2) CBE has 1:1 (mole/mole) stoichiometry with the enzyme,[59] and (3) both glucosylceramide and glucosylsphingosine hydrolytic capacities are deficient in cells from Gaucher disease patients.[133,134]

b. GLYCON, ALKYL-AMINE, AND ALKYL-GLYCON INHIBITORS

The specificity of glycon binding has been assessed by determination of apparent binding constants for a series of epimers and substituted glucose derivatives.[137] Although no particular hydroxyl group of glucose appeared necessary for interaction with the enzyme, the configuration of these groups or the introduction of charged groups did influence the apparent

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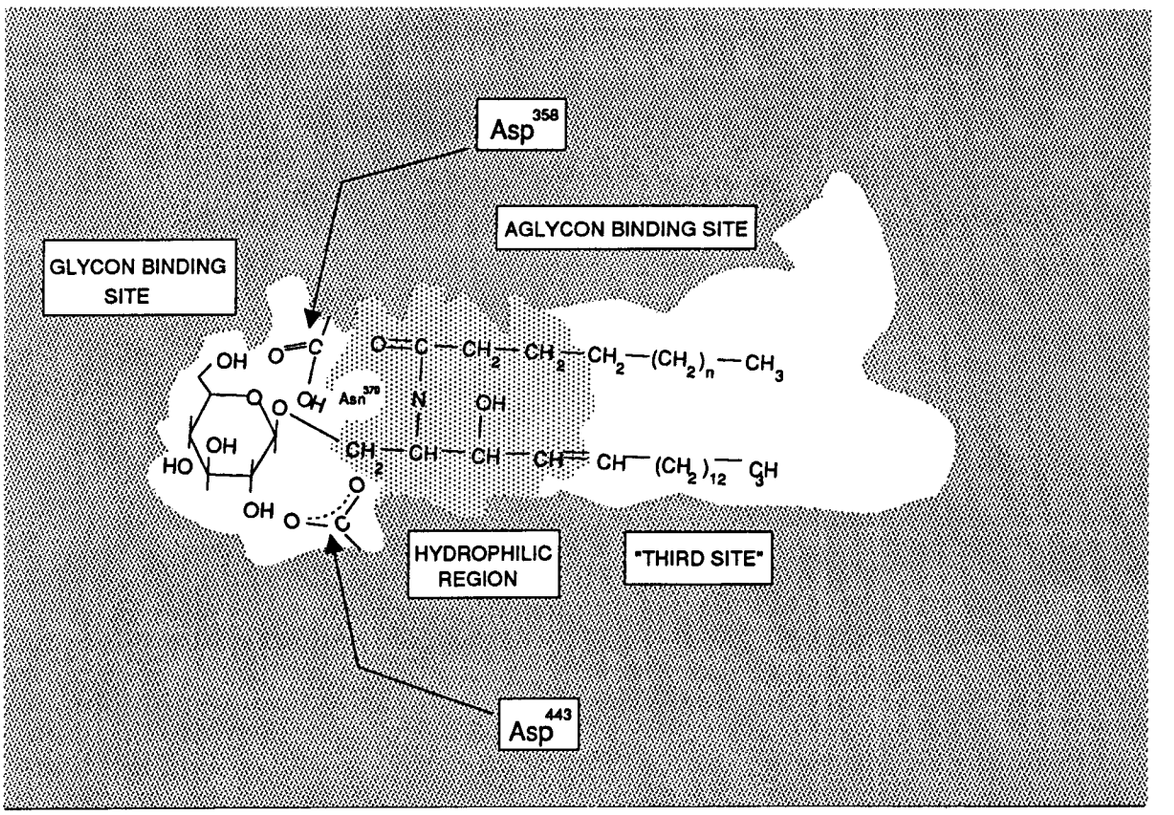

FIGURE 7. Schematic diagram for the active site of acid β-glucosidase. Aspartates 358 and 443 are shown as the proton donor and nucleophile for catalysis, respectively. Asn^{370} is the substituted amino acid in the most common Gaucher disease type 1 mutation; the $Asn^{370} \rightarrow$ Ser substitution resulting from 5481G imparts the Group B kinetic properties to the mutant enzyme. The glycon binding site has specificity for the glycon head group of substrates and inhibitors; the important amino acids for specific interaction with the C2, C3, and C4 hydroxyl groups are not indicated. The aglycon binding site and the "third site" have specificity for the fatty acid acyl chains and the sphingosyl moieties of glucosylceramide. The relatively hydrophilic region recognizes the 2-amine, 3-hydroxyl, and double bond of the sphingosyl moiety. Continuity of the amino acid sequence is not implied by the figure since the folding of the protein and the location of the residues are unknown.

binding constant, $K_i$app, relative to glucose. The C-1 anomers of glucose bound equally poorly, while the C-2, C-3, and C-4 epimers (α- or β-mannose, D-allose, or β-galactose, respectively) were very poor inhibitors (K_i>250 to 500 m*M*). Major increases of inhibitory potency (2 to 4 orders of magnitude) were achieved by the introduction of an amine at C-1 (β-glucosylamine) and C-3 (3-deoxy-3-amino-glucose) or a nitrogen at the oxygen of the pyranose ring (5-deoxy-5-imino-glucose derivatives). The latter compounds, which were derivatives of nojirimycin, had $K_i$app values of 5 to 50 μ*M* compared with K_i = 220 m*M* for β-glucose. The specificity of the active site was indicated, since only the gluco derivatives of nojirimycin inhibited, i.e., the galactodeoxynojirimycin and deoxymannojirimycin even in millimolar concentrations did not inhibit the enzyme.[137] Evaluation of the pH variation of $K_i$app for the nojirimycin derivatives and accounting for the ionization of their imino groups, these investigators concluded that the results were consistent with the unprotonated form of these inhibitors binding to the protonated forms of the enzyme (EH and EH_2) and that an ionizable group with pK_a 6.7 was important to their binding. These results suggest that although these were potent inhibitors, they were not reaction intermediate analogs (i.e., transition state analogs), since they bind to both active and inactive enzyme forms. In this respect, human acid β-glucosidase was similar to the β-glucosidase from sweet almonds.[144]

The finding that sphingosine was a potent inhibitor of acid β-glucosidase led to the investigation of its structure/activity relationships. For such studies, Hyun and Radin[141] evaluated the inhibitory potencies of a series of *N*-alkyl-3-phenyl-2-amino-1,3-propanediols. These compounds share some polar head group similarity with sphingosine, but contain a 3-phenyl in

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place of the 1-hydroxy of sphingosine. In addition, they lack the C4-C5 *trans* double bond of sphingosine. Nonetheless, these investigators found that the DL-*erythro* derivatives were much more potent than the corresponding *threo* analogs. More recently, Greenberg et al.[136] evaluated structure/activity relationships using a series of synthetic sphingosine analogs of varying chain lengths and compositions (Figure 3). Maximal inhibitory potency was obtained with the 14 carbon derivative and the *threo* configuration at carbon 2 had no major effect on the IC50 values when compared with the natural *erythro* derivatives. Unsaturation at the C4-C5 bond was necessary for inhibition.[135,136] These results indicate that the active site of acid β-glucosidase can accommodate sphingosyl moieties based on the number of methylene groups present up to about 18. The importance of *trans vs. cis* configuration at the C4-C5 double bond has not been examined.

Since the alkylamines and the nojirimycin derivatives were effective inhibitors of acid β-glucosidase, it was reasonable to suspect that an alkyl-glycons might be extremely powerful inhibitors and potentially reaction intermediate analogs. Indeed, the alkyl-1-*O*-β-D-glucosides inhibited human acid β-glucosidase with $K_i$app values that varied as a function of alkyl chain length: the dodecyl derivative was most potent ($K_i$app = 150 μ*M*).[44,45,137] Similarly, the $K_i$app of the *N*-alkyl-deoxynojirimycins (Figure 3) was dependent directly upon the length of the alkyl chain[43,44] and that inhibitory potency conformed to a model that assumed additivity of the additional free energy of binding of the alkyl chain and the glycon head group.[44] However, large increases in inhibitory potency were found only with alkyl chains greater than four carbons and limiting values were approached with the 12 and 14 carbon derivatives.[43,44] This result indicated that the binding site for the alkyl chain was separated from that for the polar glycon head group by the equivalent of about 4 to 6 carbon bonds. The "gap" was proposed to be a relatively hydrophilic region in the active site for interaction with the polar head group of sphingosine (Figure 7).[43,44]

The use of *N*-alkyl-glucosylamines (Figure 3) as inhibitors of acid β-glucosidase has further defined the specificity of binding at the active site and provided insight into this enzymes' mechanism of action.[136] Compared with the *N*-alkyl-deoxynojirimycins, the corresponding glucosylamine analogs were 20 to 100 fold more potent inhibitors. In addition, this small change of placement of the nitrogen and alkyl chain from the 5 position in the pyranose ring to the anomeric carbon altered the nature of the inhibition. The inhibitory potency of the *N*-alkyl-glucosylamines was directly dependent upon the concentration of acid β-glucosidase, i.e., they were tight binding inhibitors. Furthermore, using the 4MU-Glc in a phosphatidylserine-based system, progress curves for this substrate's hydrolysis in the presence of *N*-alkyl-glucosylamines demonstrated that full inhibition or release from inhibition was achieved only after 2 to 5 min[136] (Figure 8), i.e., they were slow-tight binding inhibitors as defined by Morrison and Walsh.[145] Analyses of these progress curves indicated that these extremely potent inhibitors induced a conformational change, an isomerization, of the enzyme after the formation of the initial collision complex according to Scheme 1.

$$E \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EI^* \qquad \text{(Scheme 1)}$$

The overall inhibitory constants were about 100 to 500 p*M*. These results were consistent with the *N*-alkyl-glucosylamines being reaction intermediate analogs and with the catalytic mechanism requiring a conformational change in the enzyme during substrate hydrolysis.

c. CONDURITOL B EPOXIDE DERIVATIVES

Conduritol B epoxide (CBE), 1-D-1,2-anhydro-*myo*-inositol (Figure 9), derivatives are active site-directed inhibitors of many β-glucosidases and covalently bind to this site via the formation of an ester linkage to a catalytic nucleophile.[146] Radioactively labeled CBE derivatives have been used to identify active site amino acid residues in several β-glucosidases,[59, 146-148] including the human acid β-glucosidase.[59] Invariably this amino acid is an asparatate. Using a specific immunoprecipitation technique, Dinur et al.[59] demonstrated a 1:1 (mole/mole) stoichiometry between homogeneous acid β-glucosidase from human placentae and [^{3}H]-Br-CBE. Subsequent experiments[59,149] using solid phase amino acid sequencing demonstrated that all recoverable radioactivity from [^{3}H]-Br-CBE bound to acid β-glucosidase peptides was associated with Asp.[443] Binding of CBE derivatives to this residue in the active site of human acid β-glucosidase seems to proceed by a two-step process.[46] (1) Initially a non-covalent, reversible binary complex is formed and (2) a subsequent acid-catalyzed step—a proton donation to the epoxide ring oxygen assisting ring opening and the formation of a carbonium ion at C1, which is attacked by the catalytic nucleophile, Asp[443] to form an ester bond. The fact that the mutant allele for β-Glc-$^{Asp358\rightarrow Glu}$ expressed a stable, but inactive, enzyme indicates a critical role for Asp[358] in catalysis, possibly the proton donor.[138] The proton donor and the nucleophile, involved in CBE inhibition, are presumed to be the properly aligned acid and nucleophile that participate in substrate catalysis.[150] CBE derivatives are likely to participate in many of the same steps as substrate hydrolysis. However, the product release step cannot be assessed. Consequently, determinations of the maximal rates of inactivation of acid β-glucosidase by CBE provide assessments of the integrity of the catalytic rate constants for substrates up to the release of the glycon product from the enzyme.[150] Although the reaction mechanism for acid β-glucosidase substrate hydrolysis is poorly defined, a potential

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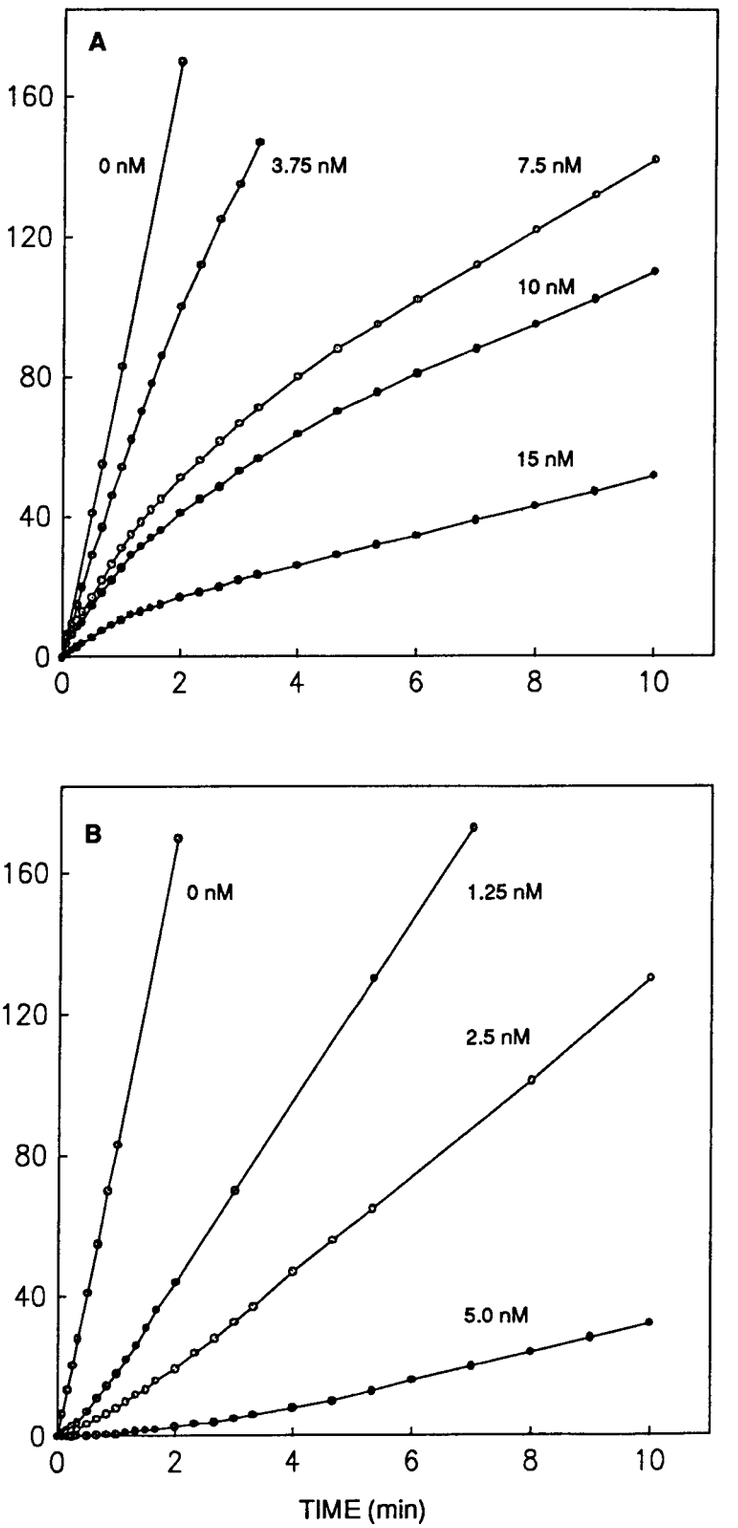

FIGURE 8. Progress curves for the hydrolysis of 4MU-Glc in the presence or absence of *N*-C_{16}-glucosylamine. In A the assays were initiated by the simultaneous addition of pure acid β-glucosidase and inhibitor into the substrate solution. In B concentrated enzyme and inhibitor were preincubated together prior to rapid dilution into a substrate solution. The final concentrations of the inhibitors are noted and the enzyme concentration was 1.1 n*M*.

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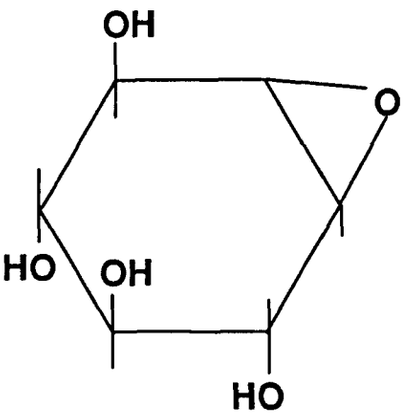

FIGURE 9. Conduritol B epoxide, 1-D-1,2-anhydro-*myo*-inositol, an active site-directed covalent inhibitor of acid β-glucosidase.

scheme, based on interactions with CBE derivatives, is shown in Figure 10.

F. Kinetic Studies of the Residual Acid β-Glucosidase in Gaucher Disease Sources

Several investigators have suggested groups of mutant enzymes in Gaucher disease sources based on their different phenomonological responses to the various modifiers of acid β-glucosidase activity.[41,46,105,106,120,132,151,152] Grabowski and co-workers[42,44,132,152] proposed the following nosology. Group A residual activities have normal responses to the negatively charged lipids, taurocholate or phosphatidylserine derivatives, and the inhibitors, sphingosyl, CBE, and 5-imino- or 1-amino-glucose derivatives. Group B residual activities had abnormal responses to these modifiers. Compared with Group A, the Group B enzymes had increased degrees of activation by negatively charged lipids[45,105,106,132] and 3- to 5-fold decreased affinity of the enzyme for the above inhibitors. These differences were found with either glucosylceramide or 4MU-Glc[42,44,46,105,106,132,152] substrates and in the presence or absence of SAP-2,[44,105,106,132] With the recent identification of the extensive molecular heterogeneity within and among the types of Gaucher disease, in retrospect, there was a remarkable consistency in the segregation of the Group B enzymes with type 1 Gaucher disease, particularly those of Ashkenazi Jewish descent, irrespective of their degree of clinical involvement. Expression studies have assigned the Group B characteristics to a $Asn^{370} \rightarrow Ser$ substitution, which is a common mutation in the type 1 Gaucher disease population and interferes with active site function (see Section V.D). The Group A residual enzymes were found in types 1, 2, and 3 Gaucher disease patients representing all ethnic populations. From the results of processing and molecular studies, the Group A enzymes result from several different mutations that lead to the syntehsis of acid β-glucosidases with severely decreased half-lives within cells. These studies and analyses of the mutant proteins expressed from specific alleles should lead to a detailed function map of acid β-glucosidase and, importantly, provide evidence for the causality of the various mutations identified in Gaucher disease patients.

V. MOLECULAR BIOLOGY OF ACID β-GLUCOSIDASE AND GAUCHER DISEASE

A. Genomic Organization

The complete sequences of the human acid β-glucosidase structural gene (7604 bp) and its pseudogene have been reported.[153] Both human sequences are contained within a single 32 kb fragment of genomic DNA, with the pseudogene being 3′ to the structural gene[153] and they map to human chromosome 1(q21→31).[48-50] In the regions present in both sequences, 96% nucleotide identity was found. The pseudogene contains several termination condons within its exons[153] and has a very weak promoter.[153] The pseudogene is smaller than the structural gene due to large deletions in introns 2 (313 bp), 4 (626 bp), 6 (320 bp), and 7 (277 bp) as well as two small exonic deletions (exon 9,55 bp and exon 4,5 bp) (see Figure 11). The four large deletions in the pseudogene represent *Alu* sequences flanked by direct repeats within the structural gene. The structural gene also contains an inverted *Alu* sequence in intron 2 not found in the pseudogene.[153] In addition, the pseudogene contains numerous point substitutions and deletions in introns and exons scattered throughout the sequence. Excluding the 55 bp exon 9 deletion, the homologous exons 7 to 11 contain only 13 isolated point difference. These differences and homologies have implications for the identification and characterization of mutations that result in Gaucher disease as well as the diagnostic accuracy of DNA-based tests for the disease variants (see below).

Analyses of the 5′ genomic sequences of the structural gene and the pseudogene indicated in presence of promotor elements in these regions.[154,155] The promoter of the structural gene contained two TATA boxes that lie between nucleotides (−23) to (−27) and (−33) to (−38) and two possible CAAT-like boxes between nucleotides (−90) to (−94) and (−96) to (−99) in relation to the most 5′ in-frame ATG of the mRNA.[153-155] No Sp1 binding site, i.e., the GGCGGG motiff, was found. These results are interesting since the promotor elements that contain TATA and CAT boxes have been identified most commonly in highly regulated genes.[156] Analyses of the promotor activity was monitored using a plasmid pSVOCAT constructs containing the appropriate acid β-glucosidase 5′ sequences and the bacterial CAT gene.[153-155] Following transfection of these constructs into different human cell lines expression of CAT activity indicated that the promotor sequences from the structural gene were at least eight times more potent than the corresponding pseudogene sequences.

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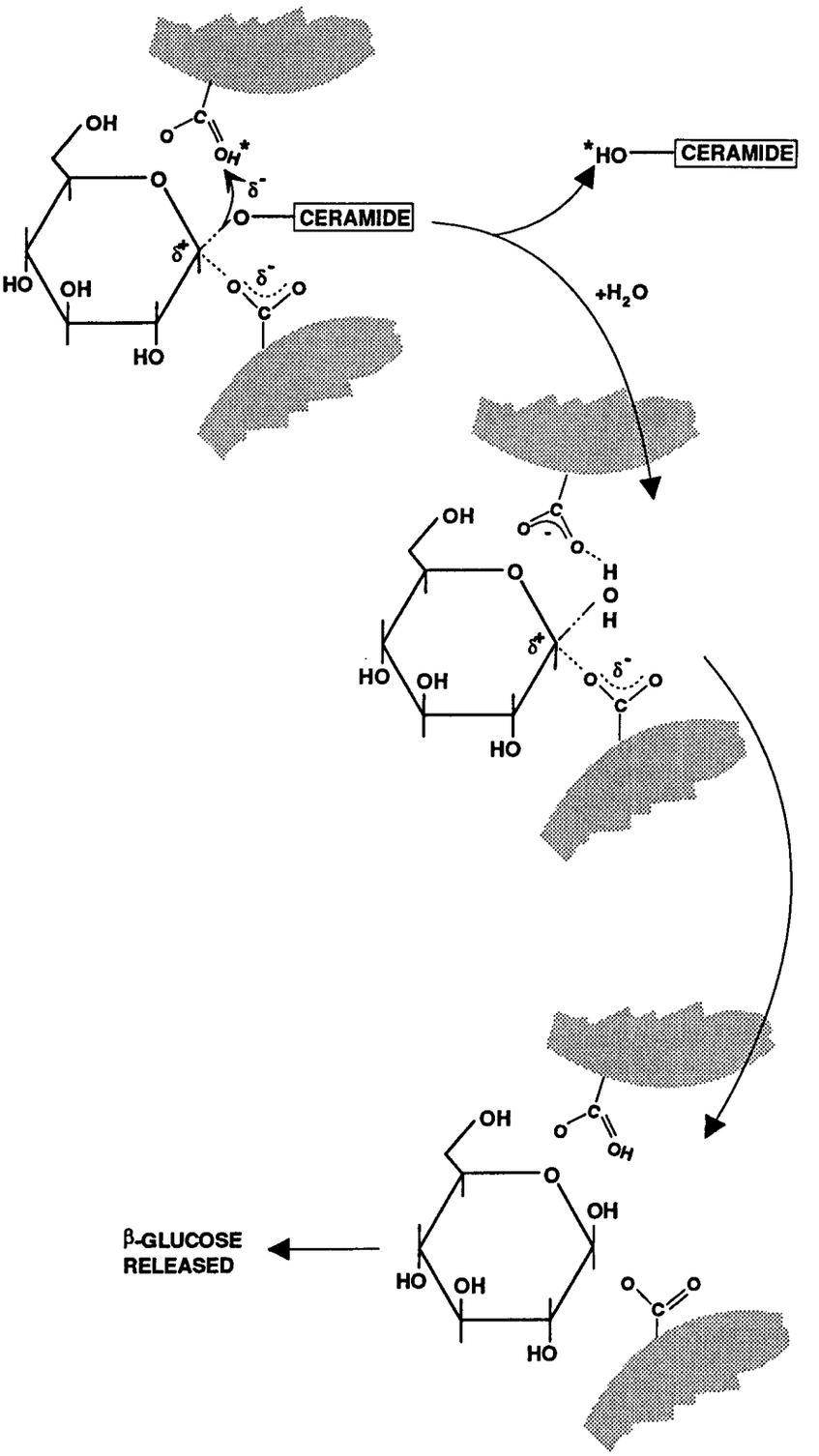

FIGURE 10. Proposed reaction scheme for the hydrolysis of glucosylceramide by acid β-glucosidase. Glucosylceramide binds to the active site and partial bonds are formed between the anomeric *O*-glycosidic linkage and a proton donor and nucleophile on the enzyme. Ceramide is released and water is added to the active site-glucosyl complex. This complex is stabilized either by ion pair formation or by a covalent linkage to Asp^{443}, the nucleophile for catalysis (second panel). Glucose then is released with retention of anomeric configuration.

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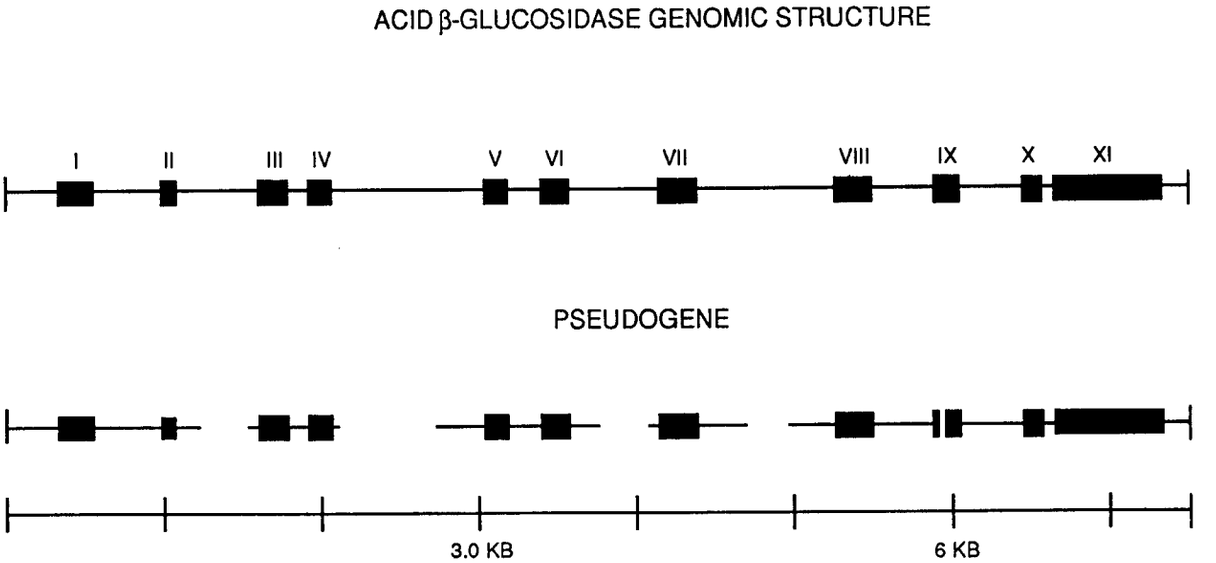

FIGURE 11. Diagram of the acid β-glucosidase structural gene (top) and pseudogene (bottom). The corresponding "exons" in each sequence are aligned and the intronic deletions are indicated. A 55 bp deletion in exon 9 of the pseudogene is also shown. Additional point mutation differences between the two sequences are not shown.

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Since *Alu* sequences are believed to be mobile elements[157,158] that may be inserted into DNA, it is possible that the pseudogene more closely resembles the ancestral acid β-glucosidase gene and that the current structural gene has been modified by insertion of these sequences. The proposed mechanism of insertion of *Alu* sequences[159,160] results in duplication of a sequence present in single copy in the ancestral gene to form the long direct repeats that flank the *Alu* sequences. Indeed, the flanking repeats found in the acid β-glucosidase structural gene are represented as single copy in the pseudogene. Since the mouse has only one gene,[56] it seems plausible that during evolution the structural gene was duplicated to give rise to two genes, one that has undergone numerous mutations and became inactive while the structural gene has undergone non-lethal evolution. It is important to note that only a single pseudogene sequence has been determined and that other forms may exist.

The mouse acid β-glucosidase gene has been cloned and its genomic organization determined.[56] This gene maps to mouse chromosome 3.[56] Following optimal alignment, the exons of the two genes have been shown to be very homologous with 84% overall nucleotide identify in the protein coding, 54% in the 5′ non-coding, and 78% in the 3′ non-coding regions. The positions of the 20 intron/exon junctions in the structural genes from each species were precisely conserved (see Figure 4 for schematic representation). Compared with the human sequence (1) a single amino acid deletion, His^{273} was predicted in the mouse sequence, (2) 64% of the nucleotide substitutions resulted from third position changes, (3) 100% of the cysteines, 91% of prolines, and 89% of glycines were preserved, and (4) the greatest variation occurred in the N-terminal 60% of the predicted amino acid sequences. Using the PAM matrix of Dayhoff,[161] O'Neill et. al.[56] indicated that all six uncommon amino acid substitutions as well as the His^{273} deletion occurred in this region of the predicted mature protein sequence. If analyzed on the basis of percentage of the mature protein sequence per exon compared with the percent variation per exon, the expected ratio of these parameters for random divergence would be about 1. When calculated from the published murine and human cDNA sequences, the 65 predicted non-conservative amino acid differences gave a ratio about 1 to 1.5 for all exons except exons 8 and 9, where the ratios were 0.2 and 0.4, respectively. This result and the carboxy-terminal conservation suggested conserved functions for selected portions of these genes. Additional support for the location of important residues for acid β-glucosidase function and, in particular, active site function is suggested by the assignment of ASP^{443} as the nucleophile for catalysis and the fact that many of the naturally occurring mutations in the human structure gene that alter acid β-glucosidase function and are causal to Gaucher disease have been found in exon 9 and 10.[59,149]

B. mRNA and cDNA Structure, Organization and Expression

Northern analyses of poly $(A)^+$ RNAs from Hela (epithelioid cells) and cultured skin fibroblasts from normal individuals and Gaucher disease patients indicated the presence of three acid

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β-glucosidase specific mRNAs of ~5.6, 2.5, and 2.0 kb in length.[154,162] The longest mRNA was thought to represent an unspliced or partially spliced nuclear transcript, whereas the shorter mRNAs were suggested by S1 nuclease analyses to arise from alternate transcription initiation as well as alternative polyadenylation.[162] Use of alternative polyadenylation signals about 450 bp apart was demonstrated by sequence analyses.[155] In addition, primer extension studies[154,155] demonstrated that the full-length cDNA extends at least 237 bp 5′ of the most 5′ ATG. These results indicate that the major full-length mRNA is about 2.5 to 2.6 kb in length and has about 250 bp of 5′ and 550 bp of 3′ untranslated sequence as well as 100 bp poly (A) tail.[154,163]

An unusual feature of the human acid β-glucosidase cDNA was the presence of two 5′ in-frame ATGs as possible translation initiation condons.[53-55,163] These two ATGs begin 57 and 118 nucleotides 5′ to the first nucleotide of the codons for the mature polypeptide (Figure 4). The possible translation products encoded by sequences downstream from these ATGs differed markedly. If translation initiated from the most 5′ ATG, a very hydrophilic, charged sequence of 19 amino acids in length would be followed by a typical hydrophobic signal sequence of 20 amino acids in length, which begins at the second ATG. This downstream ATG is preceded by a Kozak consensus sequence for translation initiation. Both ATGs were efficiently translated in a cell-free system.[155] Site directed mutagenesis and expression studies of cDNAs containing either one or both ATGs demonstrated that functional acid β-glucosidase of the appropriate size was synthesized from these retroviral constructs, which has been transfected into human Gaucher disease fibroblasts or NIH3T3 cells.[164,167] These results and expression of functional acid β-glucosidase form cDNAs coupled to the SV40 early promoter in COS cells[54,165] or in appropriate constructs in insect cells[165,166] provided demonstration of the authenticity of these cDNAs.

C. Expression of Acid β-Glucosidase mRNA

Consistent with the presence of residual acid β-glucosidase activity in nearly all Gaucher disease patients, no gross rearrangements of the mRNA from patients were detected by S1 nuclease analyses.[149,162] The level of mRNA in Gaucher disease fibroblasts was estimated to be normal within a factor of about 2,[162] but more recent quantitative studies suggest that the mRNA levels were increased by about 2- to 3-fold in cultured fibroblasts and lymphoid lines from Gaucher disease patients.[54] In addition, epithelial cells expressed high levels of acid β-glucosidase mRNA, while skin fibroblasts and promyelocytes had intermediated steady-state levels.[54] Macrophages had low and B-cells had barely detectible steady-state levels of acid β-glucosidase mRNA. Consistent with these results were the parallel levels of CAT activity expressed from constructs containing the acid β-glucosidase promoter in the corresponding tissues.[54] These results suggest the involvement of a feedback regulation of acid β-glucosidase expression.

D. Mutations in the Acid β-Glucosidase Gene Resulting in Gaucher Disease

Interest in the genetic mutations causal to Gaucher disease derives from the need to explain the marked heterogeneity of phenotypes within and among the variants. Since the level of residual enzyme activity or the properties of the mutant enzymes within cells from Gaucher disease variant patients have not consistently correlated with the severity phenotype, the need for genetic markers of disease severity[168,169] has had increasing practical (i.e., prognostic) and basic (structure/function) importance. The point mutations described to date have been identified by sequencing across exons in genomic clones[51,170] or sequencing complete cDNAs from constructed libraries from Gaucher disease patients.[54,154,171,172] These are summarized in Figure 4 and Table 2. Following the identification of a mutation, their presence in populations of patients and their frequencies were determined by allele specific oligonucleotide probing of genomic DNA or amplified genomic fragments or by restriction endonuclease analyses.[168,175] For the acid β-glucosidase gene the major complicating and significant problem for the diagnostic implementation is the presence of the highly homologous pseudogene and the presence of structural gene alleles bearing multiple-point mutations. Since PCR amplification of genomic sequences has supplanted the use of unamplified DNA, methods for selective amplification of the structural gene sequences have exploited the presence of the exon 9 deletion in the pseudogene for analyses of exon 9 and 10 mutations, which appear to be the most common.[168-175] From studies on large populations of Gaucher disease patients, two mutations in exon 9 (5841G, $Asn^{370}\rightarrow Ser$) and exon 10 (6433C, $Leu^{444}\rightarrow Pro$) account for about 60% of all alleles present in the heteroallelic or homoallelic states in all patients. Importantly, two studies have demonstrated that the homozygous presence of the 5841G is correlated with a milder phenotype and longevity.[168,169] Furthermore, no patient with neuronopathic disease has been found to have, even in single copy 5841G.[168,169,172] Although these results require additional confirmation, sufficient families have been studied to indicate that the presence and prognosis of nonneuropathic Gaucher disease can be predicted in many families on the basis of genotyping.

A puzzling finding in several studies[168,172,175] was the apparent homozygous presence of 6433C alleles in both the very acute neuronopathic, type 2, and the subacute neuronopathic, type 3, patients. However, complete sequencing of the cDNAs representing both alleles from type 2 patients with apparent 6433C homozygosity revealed the presence of additional point mutations on at least one allele. These multiply mutated alleles have been termed "complex A" (Table

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Table 2
Mutations of the Acid β-Glucosidase Gene in Gaucher Disease

Disease phenotype	Nucleotide number		Base change	Designation	Exon	Amino acid change	Enzyme abbreviation
	cDNA	Genomic					
Frequent alleles							
1	1226	5841	A → G	5841G	9	Asn^{370} → Ser	$\beta\text{-Glc}^{Asn370\rightarrow Ser}$
2 and 3	1448	6433	T → C	6433C	10	Leu^{444} → Pro	$\beta\text{-Glc}^{Leu444\rightarrow Pro}$
Rare alleles							
1	476	3060	G → A	3060A	5	Arg^{120}–Cln	$\beta\text{-Glc}^{Arg120\rightarrow Gln}$
1	535[a]	3119	G → C	3119C	5	Asp^{140}–His	$\beta\text{-Glc}^{Asp140\rightarrow His}$
	1093[a]	5309	G → A	1093A	8	Glu^{328}–Lys	$\beta\text{-Glc}^{Glu328\rightarrow Lys}$
1	580	3164	A → C	3164C	5	Lys^{155}–Gly	$\beta\text{-Glc}^{Lys155\rightarrow Gly}$
1	764	4113	T → A	4113A	7	Phe^{216}–Tyr	$\beta\text{-Glc}^{Phe216\rightarrow Tyr}$
2	1090	5306	G → A	5306A	8	Gly^{325}–Arg	$\beta\text{-Glc}^{Gly325\rightarrow Arg}$
2	1141	5357	T → G	5357G	8	Cys^{342}–Gly	$\beta\text{-Glc}^{Cys342\rightarrow Gly}$
1 and 3	1297	5912	G → T	5912T	9	Val^{394}–Leu	$\beta\text{-Glc}^{Val394\rightarrow Leu}$
1	1342	5957	G → C	5957C	9	Asp^{409}–His	$\beta\text{-Glc}^{Asp409\rightarrow His}$
3	1343	5958	A → T	5958T	9	Asp^{409}–Val	$\beta\text{-Glc}^{Asp409\rightarrow Val}$
2	1361	5976	C → G	5976G	9	Pro^{415}–Arg	$\beta\text{-Glc}^{Pro415\rightarrow Arg}$
1 and 2	1505				10	Arg^{463}–Cys	$\beta\text{-Glc}^{Arg463\rightarrow Cys}$
Complex alleles							
1 and 2	1448	6433	T → C	6433C	10	Leu^{444}–Pro	
	1483	6468	G → C	6468C	10	Ala^{456}–Pro	$\beta\text{-Glc}^{COMPLEX\ A}$
	1497	6482	G → C	6482C	10	Val^{450}–Val	
3	1141	5957	G → C	5957C	9	Asp^{409}–His	
	1448	6433	T → C	6433C	10	Leu^{444}–Pro	
	1483	6468	G → C	6468C	10	Ala^{456}–Pro	$\beta\text{-Glc}^{COMPLEX\ B}$
	1497	6482	G → C	6482C	10	Val^{460}–Val	

[a] Both mutations found on the same allele. The other allele in this patient contained only the 3164C mutation.

2).[170] Consequently, these type 2 patients had two different alleles, one having only the 6433C mutation and the other having a more severely mutated allele with an additional exon 10 missense (6468C) mutation.[170] This complex allelea, termed $\beta\text{-Glc}^{COMPLEX\ A}$, has been found in type 1 patients,[170,175,176] but only in association with the 5841G allele. Only those patients with 6433C and $\beta\text{-Glc}^{COMPLEX\ A}$ alleles had type 2 disease and true homozygosity for 6433C was found in type 3 or very severe, young type 1 (tentative) patients.[170] Indeed, the common allele for the inbred Norbottnian type 3 variant was identified as the 6433C substitution by complete sequencing of both alleles from affected patients. This result was consistent with the presence of a *Msp* II RFLP, which had concordant segregation with this disease variant. The 6433C mutation results in the acquisition of *Msp* II and *Nci* I sites in exon 10. Another complex allele, $\beta\text{-Glc}^{COMPLEX\ B}$, which contains four point mutations (Table 2) has been described. It is likely that the complex alleles are the products of recombination events between the structural gene and pseudogene.[174]

As indicated in Table 2, a substantial number of rare mutations, i.e., restricted to a single or few families, already have been identified and additional mutations are clearly present within and among the variant phenotypes. However, expression studies of mutant alleles are required to provide evidence for causality of these mutations by accounting for the presence of residual enzymatic activity and the defects in kinetic and/or processing properties of the mutant enzymes (see Sections III.C and IV.E). Sufficient expression levels of the normal and mutagenized acid β-glucosidase cDNAs have been achieved using the baculovirus expression system to permit such comparative analyses. Glycosylated acid β-glucosidase with intact active site function,[165] and normal signal peptide cleavage[165,166] was expressed from the normal cDNA that included only the more 3′ or both of the 5′ ATGs. Importantly, using a plasmid (pAc610) that provided relatively low level expression of acid β-glucosidase, Grabowski et al.[165] used kinetic and immunologic methods to demonstrate that the expressed enzyme retained full catalytic activity.

Although suboptimal for the industrial production of acid β-glucosidase, the lower level expression systems based on plasmid pAc610 have provided a convenient system for the eval-

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uation of the effects of various mutations in the acid β-glucosidase sequence. Grace et al.[60,139] have expressed several β-glucosidase cDNAs containing individual mutations for two mutations found in the Ashkenazi Jewish Gaucher disease type 1 population, $Arg^{120}\rightarrow Gln$(3060A) or $Asn^{370}\rightarrow Ser$(5841G) as well as a mutation that arose during cDNA synthesis, $Asp^{358}\rightarrow Glu$. As assessed by immunoblotting, substantial amounts of acid β-glucosidase protein were expressed from each of these cDNAs, but only the enzyme containing the Asn^{370} to Ser^{370} mutation was catalytically active toward the 4MU-Glc and glucosylceramide substrates.[139] Extensive kinetic analyses indicated that the enzyme expressed from this cDNA had the abnormalities that had been assigned to Group B residual enzymes in Ashkenazi Jewish Gaucher disease type 1 patients, i.e., abnormal fold-activation by negatively charged phospholipids, 3- to 5-fold decreased affinity for sphingosine, deoxynojirimycin, or CBE derivatives.[139] In addition, the catalytic rate constant for $\beta\text{-Glc}^{Asn370\rightarrow Ser}$, based on the amount of cross-reacting immunologic material, was decreased to the same extent.[139] This result was similar to that obtained by active site quantitation studies using partially purified enzymes from spleens of normal individuals or homozygotes for the allele encoding $\beta\text{-Glc.}^{Asn370\rightarrow Ser}$ [46,139] Indeed, the presence of at least one allele encoding this mutation was sufficient to confer the Group B characteristics to the residual enzyme in cells from Gaucher disease patients.[139] Additional kinetic studies of $\beta\text{-Glc}^{Asn370\rightarrow Ser}$ were consistent with this substitution interfering sterically with the binding of selected amino glycons and their *N*-alkyl derivatives by a local distortion of active site conformation. In comparison, the other common Gaucher mutation, 6433C, expresses $\beta\text{-Glc}^{Leu444\rightarrow Pro}$, which has normal active site function but that is highly susceptible to proteolytic digestion.[60,62]

Although alleles other than $\beta\text{-Glc}^{Asn370\rightarrow Ser}$ may express acid β-glucosidases with the Group B properties, the enzyme expressed in *Sf9* cells from one other identified Ashkenazi Jewish Gaucher disease type 1 allele, $\beta\text{-Glc}^{Arg120\rightarrow Gln}$, was inactive toward 4MU-Glc or glucosylceramide substrates at all pH values tested, even though it was glycosylated and stable to proteolytic digestion.[139] Consistent with this result, the CRIM specific activities of the acid β-glucosidase in fibroblasts from the patient with the $\beta\text{-Glc}^{Asn370\rightarrow Ser}/\beta\text{-Glc}^{Arg120\rightarrow Gln}$ genotype were 9- and 2-fold decreased compared with those from normal or $\beta\text{-Glc}^{Asn370\rightarrow Ser}$ homozygote cells, respectively. These results imply about a 2-fold lower enzymatic activity in cells from patients with $\beta\text{-Glc}^{Asn370\rightarrow Ser}/\beta\text{-Glc}^{Arg120\rightarrow Gln}$ genotypes compared with $\beta\text{-Glc}^{Asn370\rightarrow Ser}$ homozygotes. This finding and the correlation of homozygosity for the $\beta\text{-Glc}^{Asn370\rightarrow Ser}$ allele with milder type 1 phenotypes[168,169] suggest that the nature and level of the acid β-glucosidase activity expressed from particular alleles may influence the tissue distributions, rates, and types of substrates accumulating in affected individuals. Indeed, glucosylceramide and glucosylsphingosine accumulation in the brain of an Ashkenazi Jewish Gaucher disease type 1 $\beta\text{-Glc}^{Asn370\rightarrow Ser}$ homozygote) patient was dramatically less than that in brains from type 2 patients[64] who do not have

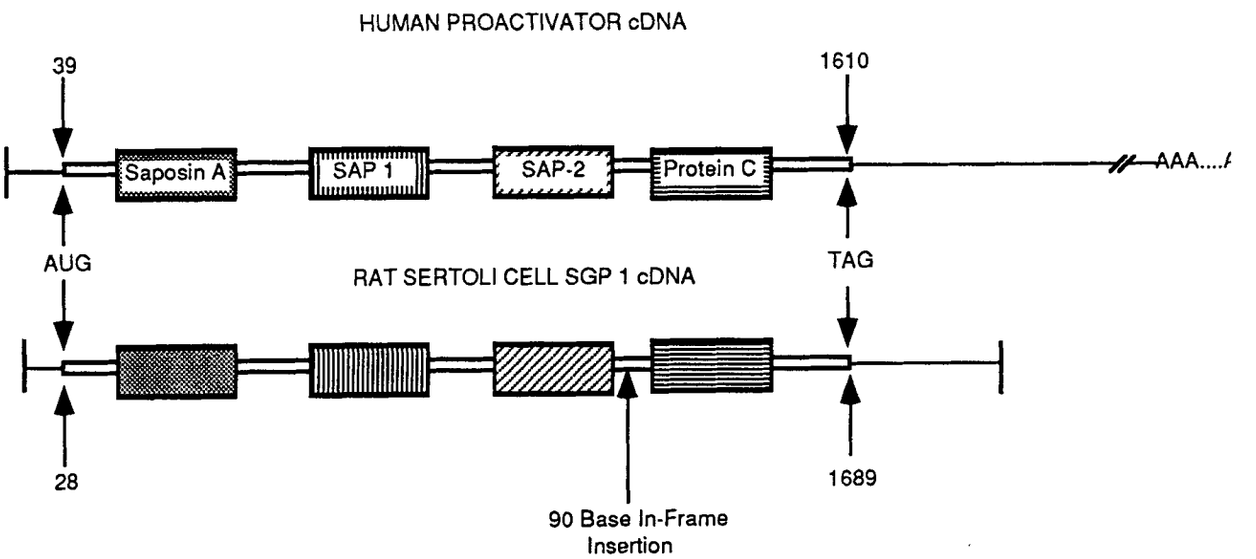

FIGURE 12. Schematic diagram of the human proactivator cDNA and the rat sertoli cell SGP-1 cDNA. The coding sequences are indicated by rectangles (open or shaded). Non-coding sequences are indicated by thin solid lines. The numbers refer to included nucleotides for the coding region. The shading rectangles indicate regions of similarity encoding functionally assigned, i.e., Saposin A, SAP-1, SAP-2, or protein C, which arise from proteolytic processing of the human proactivator molecule.

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this allele.[168,172] Continued characterization of the apparently numerous mutant enzymes expressed from different mutant β-Glcs in Gaucher disease may provide the necessary correlations to account for the markedly variable phenotypes of this disease.[168,173]

E. Molecular Biology of the Activator Proteins

Previous pulse-chasing processing studies of SAP-1 and SAP-2 indicated extensive posttranslational proteolytic processing of each from a large-molecular-weight precursor[101,102] and that the genes for both map to the same portion of chromosome 10.[177,178] The recent cloning of cDNAs encoding these "activators"[71,74-76] has demonstrated that these two "activators" as well as two additional "activators" of sphingolipid hydrolases are encoded by a single gene in humans and rats (Figure 6).[71,103] As shown schematically in Figure 12, the full-length human "proactivator" cDNA[71,76] contains regions of high amino acid similarity (>80%). The existence of these activators has been demonstrated by their partial or complete amino acid sequence determined from proteins isolated from human[70,72,179,180] or guinea pig[73] sources. The only major difference in the amino acid sequences predicted by the full-length cDNAs[71,75] was the presence of an in-frame 9 bp insertion (3 amino acids) in the SAP-1 coding sequence in the cDNAs isolated by Nakano et al.[75] from lung and skin fibroblast libraries. Furthermore, the complete chemically determined sequence of SAP-1 did not contain the predicted three amino acid insertion.[181] These results suggest a potential for tissue specific alternative splicing of the "proactivator" RNA in the SAP-1 region. Similar to the acid β-glucosidase mRNA, two species of human proactivator mRNA arise from alternative polyadenylation[76] and the levels of mRNA expression parallel that of acid β-glucosidase, i.e., the levels of proactivator mRNA were higher in normal skin fibroblasts than in B cells and the mRNA levels are higher in the corresponding cells from Gaucher disease patients.

VI. THERAPEUTIC PROSPECTS IN GAUCHER DISEASE

Gaucher disease has been considered a prime candidate for therapeutic interventions since it is the most prevalent lysosomal storage disease, its major forms are nonneuronopathic and its visceral manifestations result from bone-marrow-derived reticuloendothelial cells. Indeed, the fact that this disease has primary pathophysiology involving the monocyte/macrophage system has led to the use of bone marrow transplantation for the alleviation of symptoms in the nonneuronopathic variants[182-184] with resolution or stabilization of manifestations. The disappearance of Gaucher cells from the liver and bone marrow required as much as 2 years following transplantation and is likely related to the underlying burden of Gaucher disease cells present in these tissues at the time of transplantation. However, the morbidity and mortality of nonautologous bone marrow transplantation restricts its therapeutic usefulness to the most severely involved nonneuronopathic patients. Consequently, investigators have attempted enzyme replacement using purified placental enzyme. The initial results were difficult to interpret since enzyme was given in only a few doses due to severely limited supply of enzyme.[185] However, the recent studies have focused on the use of large doses of enzyme that has been specifically modified to expose terminal α-mannose residues on its oligosaccharide, resulting in targeting to the reticuloendothelial organs.[186] Results of these studies are very encouraging. Based on these initial results of bone marrow transplantation, enzyme replacement, and the recent successes in long-term expression of foreign genes in murine stem cells,[187,190] the future development of somatic gene transfer or more permanent enzyme replacement strategies holds the promise of hope for the thousands of affected patients with Gaucher disease.

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